



Structured Triacylglycerol of Palm-based Margarine Fat by Enzymatic Interesterification

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Structured Triacylglycerol of Palm-based Margarine Fat by Enzymatic Interesterification

By

Nuzul Amri bin Ibrahim

PhD Thesis

**BioCentrum-DTU
Technical University of Denmark
Lyngby, Denmark**

Preface

This thesis has been written in partial fulfilment of the requirement for the Ph.D. degree. Most of the work was conducted at BioCentrum-DTU, Technical University of Denmark. Some analyses were conducted at Novozymes A/S, Bagsværd. This study was sponsored by Malaysian Palm Oil Board, Kuala Lumpur, Malaysia.

I would like to express my sincere gratitude to my supervisor, Associate Prof. Dr Xuebing Xu for his kind advice, guidance and ideas. Special thanks to those who have contributed directly or indirectly to the work, especially:

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Nuzul Amri bin Ibrahim

November 2007, Lyngby

Table of Content

	Page number
Preface	2
Table of Contents	3
Abstract	7
Dansk Resumé	9
Abbreviations	11
List of Publications and Conference Presentations	13
Chapter 1. General Introduction	
1.1. Introduction	14
1.2. Interesterification	19
1.3. Objectives and Thesis Outline	20
Chapter 2. Application of Enzymatic Interesterification in Margarine Production	
2.1. Introduction	23
2.2. Margarine Production by Enzymatic Interesterification	24
Chapter 3. Margarine Fat Formulation Containing Fish Oil Assisted by Response Surface Design: Effect of Fish Oil on Physical Properties Before and After Enzymatic Interesterification	
3.1. Introduction	29
3.2. Materials and Method	
3.2.1. Materials	31
3.2.2. Methods	
3.2.2.1. TAG Analysis by GC	32

3.2.2.2. FAC Analysis by GC	33
3.2.2.3. Solid Fat Content	33
3.2.2.4. Dropping Point	33
3.2.2.5. Experimental Design	34
3.2.2.6. Enzymatic Interesterification	34
3.2.2.7. Statistic Analysis	36
3.3. Results and Discussion	
3.3.1. Enzymatic Interesterification	37
3.3.2. Model Fitting	38
3.3.3. Effect of Fish Oil on SFC For The Blend	39
3.3.4. Effect of Fish Oil on SFC for EIE Product	40
3.3.5. Effect of Fish Oil on Dropping Point	45
3.3.6. Relationship between SFC and TAG	46
3.4. Conclusion	49
Chapter 4. Enzymatic Interesterification by Multiple Lipase System	
4.1. Introduction	50
4.2. Materials and Method	
4.2.1. Materials	52
4.2.2. Methods	
4.2.2.1. Enzymatic Interesterification	54
4.2.2.2. Determination of FAC and TAG Composition	57
4.2.2.3. Evaluation Set-up for Interesterification Extent and Synergistic Effect	58

4.3. Results and Discussion	
4.3.1. Synergistic Effect of Lipozyme TL IM and RM IM	60
4.3.2. Interaction Between Lipases	61
4.3.3. Effect of Carriers on Lipozyme TL IM-catalyzed Reaction	67
4.3.4. Dual Enzyme System of Free and Immobilized Lipase	69
4.3.5. Dual Lipase System in Continuous Operation	72
4.4. Conclusion	74
Chapter 5. Improvement of Enzyme Stability with Pre-column in Continuous PBR	
5.1. Introduction	75
5.2. Materials and Method	
5.2.1. Materials	77
5.2.2. Methods	
5.2.2.1. Enzymatic Interesterification	77
5.2.2.2 Pre-column	78
5.2.2.3. Analysis of TAG Composition	78
5.2.2.4. Interesterification Degree	79
5.2.2.5. Deactivation Kinetic	80
5.3. Results and Discussion	80
5.4. Conclusion	83
Chapter 6. Overall Conclusion	84
References	86
Appendices	
Appendix I: Recycle Reactor	99

Appendix II: Triacylglycerol of Interesterified Products	103
Appendix III: Lipozyme TL IM Information Sheet	107
Paper I: Enzymatic Interesterification of Palm Stearin and Coconut Oil by a Dual Lipase System	112
Paper II: Online Pre-purification for the Continuous Enzymatic Interesterification of Bulk Fats Containing Omega-3 Oil	122
Conference Presentation I: Enzymatic Interesterification of Vegetable Oil/ Fish Oil Blend for Margarine Production	126
Conference Presentation II: Formulation of Functional Palm-based Margarine by Enzymatic Interesterification	127
Conference Presentation III: Effect of Fish Oil on Enzyme Stability during Enzymatic Interesterification	128
Conference Presentation IV: Structured Triacylglycerol of Palm-based Margarine by Enzymatic Interesterification	129

Abstract

The effect of enzymatic interesterification of a margarine formulation containing fish oil was studied by comparing the physical characteristics of the interesterified products with the same formulation without any modification process. Based on a response surface formulation design, thirteen oil blends containing palm stearin (PS) (60 to 90%), palm kernel oil (PKO) (10 to 40%) and fish oil (FO) (0 to 10%) were interesterified by Lipozyme TL IM (*Thermomyces lanuginosa*) using a continuous packed bed reactor (PBR). FO in the blend had a similar effect as PKO on solid fat content (SFC) but a stronger influence than PKO was observed in the interesterified product. Interesterified products contained higher solid fat at a temperature range from 5 to 30 °C than the blend. Enzymatic interesterification has an advantage compared to blending since the former led to products having a sharp melting point, which enabled a reduction in SFC at body temperature.

This thesis also looked into a possibility of applying a dual lipase system for interesterification of lipids. Enzymatic interesterification of palm stearin/ CnO oil blend (70/30 w/w %) was conducted by applying a mixed lipase system. Enzyme mixtures containing two types of lipases, both immobilized or combination of immobilized with non-immobilized lipase, were prepared to study their effect on the interesterification of the oil blend in batch and continuous reactors. Combination of Novozym 435 with Lipozyme TL IM and RM IM, mixture of immobilized enzymes, led to an improvement of the enzymatic activity in a batch reactor. An improvement was also observed in reaction by lipase AK Amano 20 mixed with Lipozyme TL IM, a combination of non-immobilized

with immobilized lipases. The co-immobilization action from the carrier of the immobilized lipase towards the free lipase was proposed to be one of the reasons leading to synergistic effect. No apparent synergistic effect was observed in the combination of Lipozyme TL IM and RM IM when applied to a continuous reaction.

Enzyme inactivation can be affected by polar compounds, trace elements of metals, primary and secondary oxidation products. In conducting enzymatic interesterification of oils and fats, it is important to use a good quality substrate in order to achieve the highest enzyme stability. Refined oil is preferred than crude oil since most of the compounds which caused the enzyme inactivation have been removed. Freshly refined oil contains neither oxidation products nor free fatty acid. However the oxidation products and FFA would increase over a period of time, depending on the storage condition, which could aggravate the enzyme stability. A pre-column was installed in a continuous PBR for enzymatic interesterification of sunflower oil and fish oil blend. The pre-column functions as a protector of the enzyme bed to prolong the lifespan of the enzyme. Deactivation kinetic of enzymes subjected to interesterification using a pre-column was compared against without a pre-column. The deactivation rate of enzyme bed without a pre-column was 7.4 times faster than the one with a pre-column, where the half-life were 8 and 58 days respectively.

Struktureret Triacylglycerol fra Palme-baseret Margarine ved hjælp af enzymatisk Interesterifikation

Resumé

Effekten af enzymatisk interesterificering af margarine formulaer indeholdende fiskeolie blev undersøgt ved at sammenligne de fysiske karakteristika af de interesterificerede produkter med de tilsvarende formulaer uden modifikation. Ud fra et 'response surface formulerings design', blev 13 olieblandinger indeholdende 60-90% palm stearin (PS), 10-40% palm kernel oil (PKO) og 10-40% fiskeolie (FO) interesterificeret med Lipozyme TL IM (*Thermomyces lanuginosa*) i en kontinuert 'packed bed' reaktor (PBR). FO i blandingen havde samme effekt som PKO på 'solid fat content', men en større effekt af FO var set på det interesterificeret product i forhold til PKO. Interesterificerede produkter indeholdt mere 'solid fat' i temperaturområdet fra 5 til 30 °C end de tilsvarende blandinger uden modifikation. Enzymatisk interesterifikation har en fordel i forhold til blandinger uden enzymatisk modifikation, idet førstnævnte medførte produkter med et 'skarpt' smeltepunkt, der muliggør 'SFC' reduktion ved kropstemperatur.

Thesis'en undersøger ligeledes muligheden for at anvende et 'dual lipase system' til interesterificering af lipider. Enzymatisk interesterificering af 'palm stearin/ CnO' olieblandinger (70/30 w/w %) blev udført under anvendelse af et 'mixed lipase system'. Enzym mix bestående af to typer lipaser, enten to 'immobiliserede' eller en kombination af 'immobiliseret' med 'ikke-immobiliseret' lipase blev fremstillet med henblik på at undersøge effekten på interesterificeringen af olieblandingen i såvel 'batch' som 'kontinuert' reaktor. Kombinationen af Novozym 435 med Lipozyme TL IM og RM IM og

mixturer af immobilized enzymer, førte til forbedret enzym aktivitet i 'batch' reaktoren. Forbedringer blev ligeledes set i reaktioner hvor lipase AK Amano 20 var blandet med med Lipozyme TL IM og hvor en kombination af 'ikke-immobiliseret' med 'immobiliserede' lipaser, indgik. 'Co-immobilization action' fra 'carrieren' af den ' immobiliserede' lipase overfor den 'frie' lipase var forslået som en af årsagerne til en synergistisk effekt. Der blev imidlertid ikke observeret nogen synergistisk effekt ved brug af kombinationen af Lipozyme TL IM og RM IM i en 'kontinuert' reaktor.

Enzym inaktivering kan blive påvirket af polære komponenter, sporelementer fra metaller eller primære og sekundære oxidations produkter. Ved gennemførelse af enzymatisk interesterifikation af olie og fedt er det vigtigt at benytte substrat af høj kvalitet for at opnå bedst mulig enzym stabilitet. Raffineret olie er at foretrække frem for råolie, idet de fleste af de komponenter der forårsager enzym inaktivering dermed er fjernet. Ny-raffineret olie indeholder hverken oxidations produkter eller frie fedtsyrer (FFA). Med tiden vil indholdet af oxidations produkter og FFA imidlertid stige afhængig af opbevaringsforholdene, der kan forværre enzym stabiliteten. En 'pre-column' blev tilført et 'kontinuert PBR system til enzymatisk interesterifikation af solsikkeolie og fiskeolie blandinger. Ideen med en 'pre-column' var at beskytte enzymet og dermed forlænge enzymets levetid. Deaktiverings kinetik af enzymet i forbindelse med interesterificering ved hjælp af en 'pre-column' blev sammenlignet med et system uden 'pre-column'. Deaktiveringsraten for enzymet var 7.4 gang hurtigere uden brug af 'pre-column' i forhold til brug af en 'pre-column', der havde ' half-life' på henholdsvis 8 og 58 dage.

Abbreviations

CIE	Chemical interesterification
CnO	Coconut oil
CN	Carbon number
CO	Canola oil
CSO	Cotton seed oil
DHA	Docosahexaenoic acid
DP	Dropping point
ECN	Equivalent carbon number
EIE	Enzymatic interesterification
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAC	Fatty acid composition
FO	Fish oil
GC	Gas chromatography
HLCO	High laurate canola oil
ID	Intesterification degree
LC PUFA	Long chain polyunsaturated fatty acid
MC FA	Medium chain fatty acid
MF	Milk fat
MFO	Milk fat olein
MFS	Milk fat stearin
MzO	Maize oil

N-3/ N-6	Omega-3/ Omega-6
PBR	Packed bed reactor
PKO	Palm kernel oil
PKOO	Palm kernel olein
PO	Palm oil
PR	Peak ratio
PS	Palm stearin
PUFA	Polyunsaturated fatty acid
RBO	Rice bran oil
SBO	Soya bean oil
SC FA	Short chain fatty acid
SF	Sal fat
SFC	Solid fat content
SFO	Sunflower oil
SL	Structured lipid
SMP	Slip melting point
TAG	Triacylglycerol
TFA	Trans fatty acid

List of publications

- I. Nuzul A. Ibrahim, Zheng Guo, and Xuebing Xu (2008) Enzymatic interesterification of palm stearin and coconut oil by a dual lipase system. J Am Oil Chem Soc 85:37-45
- II. Nuzul A. Ibrahim, Sanna I T. Nielsen, Vinotha Wigneswaran, Hong Zhang, and Xuebing Xu (2008). Online pre-purification for the continuous enzymatic interesterification. J Am Oil Chem Soc 85:95-98

Conference presentations list

1. A.I. Nuzul and X. Xu, Enzymatic interesterification of vegetable oil/ fish oil blends for margarine production, poster presentation at the Lipidforum seminar: Enzymes in Lipid Technology, February 26-28, 2005, Lyngby, Denmark
2. Nuzul Amri Ibrahim and Xuebing Xu, Functional margarine fat formulation containing fish oil for enzymatic interesterification, poster presentation at the Palm Oil International Congress (PIPOC 2005), September 25-29, 2005, Kuala Lumpur, Malaysia.
3. N. Ibrahim and X. Xu, Effect of fish oil on enzyme stability during enzymatic interesterification. Poster presentation at the 97th AOCS Annual Meeting, April 30-May 3, 2006, St. Louis, Missouri, USA.
4. Nuzul Amri Ibrahim and Xuebing Xu. Structured triacylglycerol of palm-based margarine by enzymatic interesterification, poster presentation at the Palm Oil International Congress (PIPOC 2007), August 26-30, 2007, Kuala Lumpur, Malaysia

Chapter 1

General Introduction

1.1. Introduction

About 150 years ago, there was a pressure in France to look for a cheaper substitute for butter to feed a growing population and armed forces. This had prompted Emperor Louis Napoleon III to offer a prize to anyone who could make a satisfactory alternative to butter. A French chemist, Hippolyte Mège-Mouriès won the prize with his invention. He named his invention margarine, originated from the Greek word 'margarites' meaning pearl, which the product resembled in appearance after being churned. Commercial production was initiated in the 1870s by the Dutch company Jurgens (Shukla, 2005). Ever since its invention, margarine manufacturing process has changed significantly from being open to the air, wet and lengthy to one that is now enclosed, dry and relatively short. Cycle times- from ingredient mixing to producing the finished packed product- have dropped from 60 hours in the early 1900s to 10 minutes by 2000 (Robinson, 2005).

Apart from the manufacturing process, margarine has undergone a lot of improvements with respect to nutritional value and physical properties to cater for the ever demanding consumers. From being a humble cheap substitute for butter, margarine has evolved to be healthier than its first invention. Currently, there are various low calorie or low fat commercial margarines and margarines fortified with vitamins and omega-3 (n-3) and omega-6 (n-6) PUFA (Flöter and Bot, 2006). The application of margarine in food has become easier with the availability of pourable margarine and readily spreadable margarine

right after it has been taken out from a refrigerator (Miskandar and Yusoff, 1998; Chrysan, 2005).

Most native vegetable oils have only limited applications in their original forms due to their specific compositions. Several modification processes are available to widen the application scope of the oil namely hydrogenation, interesterification (chemical and enzymatic), blending and fractionation (Senanayake and Shahidi, 2005). Liquid oils are prone to oxidation due to the high PUFA content and have very limited applications for margarine and shortening. It was a common practice to partially hydrogenate liquid oils to turn them into semi-solid oils for margarine hard-stock. Since the partially hydrogenated oils become more saturated, they are more stable against oxidation. However, this modification process is no longer popular for food applications since partially hydrogenated products have been proven to be detrimental to human health due to the formation of trans fatty acids (TFA) (Katan et al., 1995; Willet et al., 1993). In January 2004, Denmark imposed a regulation restricting TFA content in food, which should not be more than 2% (Jensen, 2005). USA and part of Latin America implemented labelling regulation for TFA on food products in January and August 2006 respectively (Moss, 2005; Biotimes, 2006). The restriction and labelling regulations have educated consumers to be aware of what they consume. Those regulations have also prompted food industries to find alternatives in producing healthier foods and trans-free fats. Palm stearin (PS), the hard fat fraction from palm oil, is an excellent substitute for partially hydrogenated oil since it does not require any hydrogenation process. Furthermore, it provides strength or structure which enhances the plasticity of the products (Aini & Miskandar, 2007). Therefore, it provides an

inexpensive raw material for the production of hard feedstock, which at a later stage can be used for production of trans-free margarine.

Beside the type of fatty acids, the molecular structure of fats also influences their function in human body. Many studies have been conducted on incorporating long chain polyunsaturated fatty acids (LC PUFA) and short or medium chain fatty acids (SC/ MC FA) into lipids. Such modified lipids are termed as structured lipids (SL). SL can be defined as lipids restructured or modified to change the fatty acid composition and/ or their positional distribution in the glycerol molecule (Jacobsen et al, 2006). SL can be produced by chemical and enzymatic interesterifications.

Specific lipases have contributed to the development of SL to produce new products (Xu et al., 1998a & 2002; Yankah & Akoh, 2000; Yang et al., 2001; Lee & Akoh, 1997). Therefore, EIE has increased the value of the existing oils and fats by transforming them into value-added products. Even though the process is normally associated with acyl migration, the problem can be minimized by taking a proper control of the operating condition (Xu et al., 1998b). The versatility of specific lipase has inspired the incorporation of essential FA such as eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) into lipids (Fajardo et al., 2003; Nascimento et al., 2004; Xu et al., 2000). Sundstrom et al. (2006) reported that supplementation of n-3 fatty acid in patients with ankylosing spondylitis has improved the patients' condition.

World Health Organization has recommended that the n-6: n-3 PUFA ratio should be within the range of 5:1 to 10:1 (WHO/ FAO, 1995). However, a typical western diet contains higher n-6 PUFA than the recommended range, which is up to 15:1 (Trautwein, 2001). Increment of n-3 PUFA intake would decrease the n-6 to n-3 ratio, which will be beneficial for individuals suffering from coronary heart diseases, type 2 diabetes, hypertension, immune response disorders and mental illness (Djordjevic et al., 2005). The benefits of n-3 PUFA have prompted several researchers to study the effect of n-3 PUFA enriched margarine on human (Sørensen et al., 1998, Saldeen et al., 1998).

The main objective of this project is to produce trans-free margarine hard stock fortified with n-3 fatty acids via enzymatic interesterification process. Not only the product is safe for consumption, but it is also contains essential fatty acids and thus makes it more nutritional than the traditional margarine. The objective is parallel with MPOB's strategy, which is the main sponsor of this study. Consumption of the trans-free margarine fortified with n-3 fatty acids will reduce the gap of the n-6 to n-3 ratio in the western diet.

In natural vegetable oil, most saturated fats are located at sn-1 and 3 positions while unsaturated fatty acids at sn-2 position (Bornscheuer et al., 2003). In its natural form, fish oil (FO) contains about 20 to 30% of EPA/DHA and most of the acids are located at sn-2 position on the glycerol backbone (Hjaltason and Haraldsson, 2006). In this study, FO was added into a margarine hard stock and interesterified by a sn-1, 3 specific enzyme. The specific lipase was expected to intra- and interesterifies those fatty acids at the outer positions and retains the EPA/DHA at the sn-2 position so that the interesterified product

would still maintain the nutritional attribute of the omega-3 fatty acids. By exploiting the specific lipase, the triacylglycerol (TAG) of enzymatically interesterified PS/ palm kernel oil (PKO)/ FO blend will have a different profile from the non-reacted blend, especially at the sn-1 and -3 positions. Palmitic acid (from PS) at the sn-1 and -3 will be replaced with short or medium chain fatty acids (from PKO) or PUFA (from fish oil), while EPA and DHA at the sn-2 are retained. This is just a simple illustration, where as in the actual situation, the replacement of the fatty acids is more complicated due to the presence of a wide range of fatty acids in the blend which form a complex matrix. Even though blending is a simple modification process where a desired melting point can be achieved by blending two or more oils, the TAG profiles still remain the same. On the other hand, enzymatically interesterified products have a different TAG profiles than the blend, and the rearrangement of fatty acids in the glycerol backbone causes a change in the physico-chemical properties of the blend.

Enzymatic process is widely used in high value market segment since the expensive product could compensate the high production cost. Even though its application in food industry is lagging, the scenario is slowly changing, with a few companies adopting the EIE process namely Karlshamns, Sweden (January 2001), ADM, USA (July 2002), KMT, Ukraine (January 2004) and Flora Danica, Argentina (July 2005) (Biotimes, 2006). Possible reason for the growing interest in enzymatic interesterification application by the industries could be partly due to reduction in enzyme cost through genetic engineering and commercially available immobilized enzyme which simplifies the enzyme recovery process

for recycling. Unlike free enzyme, immobilized enzyme can be applied in continuous reaction which takes shorter time than batch reaction; this translates to higher out-put.

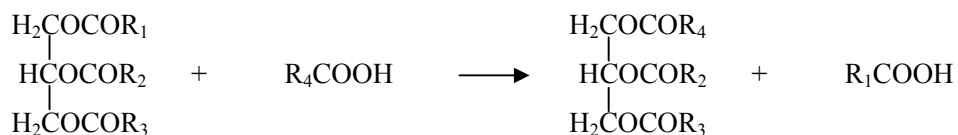
1.2. Interesterification

Interesterification is a chemical process which causes a fatty acid redistribution within and among TAG molecules, which leads to substantial changes in lipid functionality. There are two types of interesterification processes: chemical and enzymatic. Both processes can be further divided into four classes of reactions: acidolysis, alcoholysis, glycerolysis and transesterification. Acidolysis involves the reaction of a fatty acid and a TAG while alcoholysis involves the reaction of an alcohol and a TAG. Glycerolysis is an alcoholysis reaction in which glycerol acts as the alcohol. Transesterification involves the exchange of fatty acids between two TAGs to form new TAGs. Figure 1.1 illustrates the three classes of reactions.

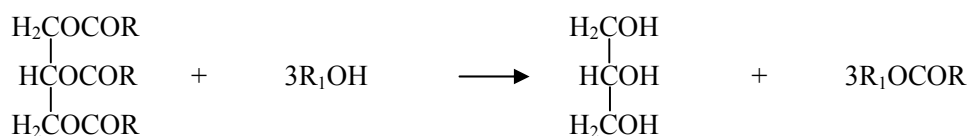
Both chemical and enzymatic interesterification (CIE and EIE) cause a re-arrangement of FAs in TAG molecule which leads to a product having a different physical property than the original stock. CIE is a matured technology whilst EIE is getting more popular due to the availability of low-cost enzyme. There are three categories of EIE reactions, depending on the applied lipases, namely non-specific, position specific (regiospecific) and fatty acid specific (stereospecific) (Macrae, 1983). Therefore, EIE is more versatile than CIE since the former is capable to perform both specific and non-specific reactions, but the latter is only suitable for randomization. Furthermore, EIE offers several other advantages such as

mild operating conditions and ease of control as reported in several publications (Zhang et al., 2004a and 2004b; Fomuso & Akoh, 2001; Zainal & Yusoff, 1999).

Acidolysis



Alcoholysis



Transesterification

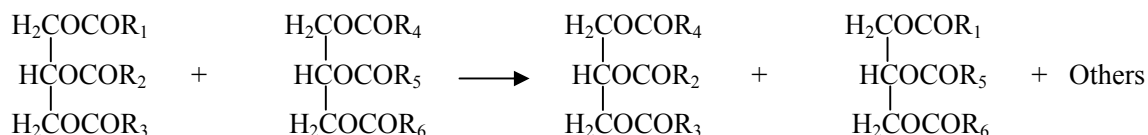


Figure 1.1. Classification of interesterification reactions. For glycerolysis, the alcohol is replaced with glycerol.

Throughout this thesis, interesterification refers to enzymatic transesterification since the substrate are palm stearin, palm kernel and fish oils which involves the exchange of fatty acids between the TAGs of the three lipids. The reaction mechanism has been explained by Xu et al. (2006).

1.3. Objectives and Thesis Outline

This study is funded by Malaysian Palm Oil Board. The sponsor's vision and mission have been incorporated to be part of the objectives of this thesis. This research would pave the

way to explore new application of palm oil and expand its usage into a value-added product.

This study focuses on producing a palm-based margarine hardstock fortified with EPA and DHA from fish oil through enzymatic interesterification process. Margarine produced from the hardstock will have a better nutritional value than normal margarine and will help to reduce the gap of n-6 to n-3 ratio in the western diet upon its daily consumption. It is important to study the effect of fish oil on the physico-chemical properties of the hardstock formulation since fish oil contains a high PUFA. The melting point and SFC of the blend will be affected and it is interesting to know the effect of enzymatic interesterification on those two properties. The objectives of this thesis also include other aspects:

1. To study the feasibility of combination of two enzymes on the interesterification.
2. To study the physicochemical characteristics of the blend and interesterified product.
3. To find ways to prolong enzyme activity

Oil blends consisting of PS (60 to 90%), PKO (10 to 40%) and FO (0 to 10%) were subjected to a continuous enzymatic interesterification using a jacketed stainless steel column (200 mm X 15 mm i.d.) filled with Lipozyme TL IM (*Thermomyces lanuginosa*) lipase, a sn-1, 3 specific lipase. The column was attached to a circulating water bath set at 70 °C. The function of fish oil was as a source for omega-3 fatty acids. The blends and interesterified products were analyzed for fatty acid composition, triacylglycerol content, SFC, free fatty acids and dropping point.

Currently, most studies have been focussing on single enzyme system. This study included an application of dual lipase system where a combination of non-immobilized/ immobilized and immobilized/ immobilized enzymes were used to see the effect of such combination on enzyme activity. PS and coconut oil (CnO) blend was chosen as a model system in this study. The blend was enzymatically interesterified in batch and continuous reactors. The synergistic effects of dual enzymes were evaluated by comparing the experimental observation of single enzyme with mixed enzyme systems.

Enzymatic interesterification is a costly process due to the high cost of enzyme. This has hampered the application of the process by industries. Furthermore, enzyme activity is adversely affected by impurities (Pirozzi, 2003). This project looked into a possibility to prolong the enzyme activity by introducing an on-line pre-filtration of substrate to remove certain degree of impurities presence in the substrate. Theoretically, a cleaner substrate would prolong the enzyme's half life. A continuous enzymatic interesterification was conducted on sunflower and fish oil blend for 200 hours at 70 °C. PUFA oils were chosen as the substrate for this experiment since they are susceptible to oxidation and operating at high temperature will accelerate the oxidation process. Residual activity of the enzyme was fitted into deactivation equation to monitor the enzyme stability by comparing the pre-filtration with the one without pre-filtration.

Chapter 2

Application of Enzymatic Interesterification in Margarine Production

2.1. Introduction

Margarine is a water-in-oil emulsion, which is visco-elastic semi-solid food product containing both liquid oil and solid fat. Previously, liquid oils were partially hydrogenated to make it more saturated and subsequently improves the oxidative stability of the oils. However, due to restriction and labeling regulations, partial hydrogenation process has been replaced by full hydrogenation, interesterification or blending with a saturated oil. These modification processes are necessary in order to achieve the desired properties of a margarine, especially the melting point and SFC. Among the three processes, blending is the simplest method which leads to a change in physical properties but it does not change the triacylglycerol molecule. The fatty acids in the TAG will remain in their original positions after two or more oils are blended. Modification by full hydrogenation also does not change the TAG.

Intesterification process will change both physical and chemical properties of an oil or blend. The process involves ester-ester exchange among and within TAG molecules and this leads to a change in the physical properties of the oil. Even though EIE is more expensive than CIE, some margarine producers have adopted the former due to several advantages it offers. Furthermore, it is claimed to be a green technology since enzymes are biodegradable protein and do not pose any hazard to the environment, unlike CIE which poses a threat in disposing the metal catalyst. No doubt, with the on-going research on EIE

to make it more lucrative, e.g. development of cheaper enzymes and improvement of enzymes stability, more companies will adopt the technology in the future.

2.2. Margarine Production by Enzymatic Interesterification

Table 2.1 shows some publications related to the application of EIE for margarine dated from 1992 to 2007. As mentioned earlier, the operating condition of EIE is milder than CIE, which is within the range of 50 to 75 °C. The reaction time varies from 15 min to 24 h, depending on the type of reaction. A continuous reaction takes shorter time than batch. Most authors applied sn-1,3 specific lipases and oil blends containing a mixture of liquid oil and saturated oil, which is a common base oil for margarine formulation. Since the substrates were a mixture of oils, most of the researchers applied interesterification while only two reported on acidolysis. Generally, all findings reported that the physical properties of the interesterified products were different from the blend.

One of the early researches on application of specific lipase is by Graille et al. (1992) where *M. miehei* was applied to interesterify blends of palm products and PO/CnO blend. The enzyme was fixed onto macroporous anion exchange resin. The author reported that the residence time for a continuous reaction affected the physical properties of the interesterified products. A firm margarine was produced when a blend of PO/PKO (30/70) was interesterified at a residence time of 30 min, but a soft margarine was obtained when the same blend was interesterified at 3.5 h. It was also reported that an enzymatically interesterified PUFA enriched PS (PS/SBO 30/70) had the same rheological properties as SFO margarine.

Table 2.1. Researches on margarine by enzymatic interesterification

Researchers	Enzyme	Substrate*	Reaction/ Residence time	Temperature, °C
2007				
De et al.	Lipozyme RMIM	MFO/SF MFO/PS MFS/CSO MFS/RBO	4h (B)	60
Siew et al.	Lipozyme TL IM	HPS/CnO	24 h (B)	60
2006				
Osorio et al.	Lipozyme TL IM	PS/PKO/SFO PS/PKO/n-3 FA	30 min-24 h (B) 15 min (C)	70
2005				
Zhang et al.	Lipozyme TL IM	PS/CnO	110 min (B)	70
Osorio et al.	Novozym 435	PS/SBO	2 h (B) 19 & 60 min (C)	70
2004				
Nascimento et al.	Lipozyme TL IM	PS/PKO/n-3 FA	15-105 min (B)	55-75
Zhang et al. ^a	Lipozyme TL IM	PS/CnO	30-180 min (B)	70
Zhang et al. ^b	Lipozyme TL IM	PS/CnO	20 min-24 h (B)	70
2002				
Torres et al.	Lipozyme TL IM	MzO/Tristearin	48 h (B)	45
2001				
Fomuso & Akoh	Novozyme 435	HLCO/Stearic acid	24 h (B)	55
Zhang et al.	Lipozyme IM & TL IM	PS/CnO	6 h (B)	70
2000				
Zhang et al.	Lipozyme IM	PS/CnO	6 h (B)	50-75
Lai et al. ^a	Lipozyme IM 60	PS/PKOO	6 h (B)	60
Lai et al. ^b	Lipozyme IM 60, <i>A. niger</i> , <i>R. niveus</i> , <i>M. javanicus</i> , <i>C. rugosa</i>	PS/MF	6 & 8 h (B)	60
1999				
Ming et al.	Lipozyme IM 60, <i>Pseudomonas</i> sp	PS/SFO	6 & 8 h (B)	60
Zainal & Yusoff	Lipozyme IM	PS/PKOO	6 h (B)	60

1998					
Seriburi & Akoh	Lipozyme IM 60, SP 435	Triolein/ Stearic acid	24 h (B)		55
Ming et al.	Lipozyme IM 60, <i>A. niger</i> , <i>C. rugosa</i> , <i>Pseudomonas</i> sp	PS/PKOO	6 & 8 h (B)		60
1997					
Ghosh & Bhattacharyya	<i>M. miehei</i>	PS/SFO PS/SBO PS/RBO	5 h (B)		60
1992					
Graille et al.	<i>M. miehei</i>	PS/PKO PO/CO PO/PKO	6 h (B) 30 min-5 h (C)		60

Abbreviations: B, batch reactor; C, continuous reactor. Others, please refer to page 11.

Zhang et al. has conducted an extensive study on PS/CnO blend which covered several aspects namely storage stability, effect of EIE on physical properties, small scale and large scale production (Zhang et al., 2001). According to her findings, the SFC of the interesterified products was sharper than the blend. Even though interesterification degree did not have any influence on physical properties, but the products were more β' tending with increasing degree of interesterification (Zhang et al., 2004b), which was similar to findings from Siew et al. (2007). Enzyme activity was monitored in an up-scale study from 1 kg (2000) to 300 kg pilot plant reactor (2001) and it was reported that the enzyme could retain its activity until 11 batches and 9 batches respectively. On storage stability test, the authors reported that a higher conversion degree led to a better stability (2005). PV value was still within the acceptable level even after three months of storage, which was similar to Lai et al. (2000a) findings.

Ming et al. (1998 and 1999) and Lai et al. (2000b) compared the effect of applying different lipases in interesterification of PS/PKOO and PS/MF. It was reported that interesterified products from *Pseudomonas* sp. had the biggest SMP drop as compared to the other lipases (please refer to Table 2.1 for lipase list). It was also suggested that the positional specificity of lipases may not play a significant role in producing a more fluid oil product (Ming et al., 1998).

Most of the authors in Table 2.1 applied interesterification in their work since the substrates were oil blends. However, a different approach was attempted by several researchers by applying acidolysis since stearic acid was used as the acyl donor. *C. antarctica* was used in preparing SL of high laurate-canola oil/ stearic acid blend. The blend containing 30% stearic acid showed the best match with commercial stick margarine in term of melting characteristics (Fomuso & Akoh, 2001). In an earlier work, IM 60 and SP 435 were applied to incorporate stearic acid into triolein and it was found that IM 60 gave higher incorporation at sn-2 position than SP 435 (Seriburi & Akoh, 1998).

Solvent fractionation of milk fat was employed by De et al. (2007) to get stearin and olein, which were then blended with rice bran oil (RBO), cotton seed oil (CSO), sal fat (SF) and PS. The blends were interesterified chemically and enzymatically (by *M. miehei*) for margarine preparation. Due to a wide range of penetration value, i.e. 112-145, the interesterified products could possibly be applied either for soft, table, tub or stick margarine.

Production of margarine enriched with n-3 FA has been reported by Osorio et al. (2006) and Nascimento et al. (2004) by using Lipozyme TL IM for different purposes; the former studied the operational stability of the lipase whilst the latter monitored the transesterification by the SFC. Lipase subjected to oil blend containing higher amount of PUFA was found to be less stable than the one subjected to oil blend with lower amount of PUFA (Osorio et al., 2006). Nascimento et al. (2004) has proposed an alternative way in monitoring transesterification reaction. In addition to TAG content, SFC can also be applied to monitor the transesterification reaction.

Chapter 3

Margarine Fat Formulation Containing Fish Oil Assisted by Response Surface

Design: Effect of Fish Oil on Physical Properties Before and After Enzymatic

Interesterification.

3.1. Introduction

In margarine formulation, a hard stock is normally blended with liquid oil to produce the desired physical property, especially the melting point, SFC and types of crystals. Previously, partially hydrogenated oil was the most common source for the hard stock. However, the process led to the formation of TFA, which is known to cause cardiovascular disease (Ascherio and Willet, 1997). Due to consumer awareness, labelling (Moss, 2005) and restriction of TFA content regulations (Jensen, 2005), the partially hydrogenated oil is replaced with either saturated or interesterified oils and fats.

EIE has generated a great deal of interest to produce fats with desirable functional and physical properties for food applications due to the regiospecificity of lipase (Yang et al., 2003; Xu et al., 2002 and 2000; Mu and Høy, 2002; Kim et al., 2002; Halldorsson et al., 2001). Such interesterified products are referred to as SL which are supposed to be more nutritional or healthful than the unmodified lipids. Therefore, EIE has increased the value of the existing oils and fats by transforming them into value-added products. The versatility of specific lipase has inspired the incorporation of essential FA such as EPA and DHA into lipids (Akoh and Moussata, 2001; Haraldsson et al., 2000; Fajardo et al., 2003; Irimescu et al., 2001).

The objective of this research is to study the effect of adding fish oil into palm-based margarine hardstock formulation on the physical and chemical properties of the blends and interesterified products through solvent-free enzymatic interesterification process using *Thermomyces lanuginosa* lipase. As mentioned in Chapter 1, enzymatic interesterification would rearrange the FAs in the TAG backbone, which caused the physical property of the interesterified product to be different from the blend. Beside EIE, the desired physical properties of a hard stock for margarine can also be achieved by blending but in the latter process, the FAs of each fat and oil in the blend would remain in their original forms. Therefore, even though it is possible to get the desired physical properties by blending, which is a straight forward process, but it does not change the functionality of the FAs in the blend since they are still the same as in the individual oil. Enzymatically interesterified products probably would have a different functionality than the non-interesterified blends. The effect of such rearrangement on functionality is beyond the scope of this thesis, however, it is noteworthy to study the effect of EIE by comparing the physical properties of the interesterified products with the non-interesterified blends.

In this study, PS and PKO were chosen as the hard and soft fractions respectively while FO is the source for EPA/DHA. Addition of FO into the formulation would affect the crystal form and melting property since FO is a highly unsaturated lipid and has a very low melting point. The experiment was designed by response surface methodology by assigning the oil composition (PS/PKO/FO) within a pre-defined range as the factors and SFC and dropping point as the responses. Thirteen oil blends were formulated based on the input. Formulation study would provide an insight on the effect of the oils on the physical

property, which oil has the most effect, and also the extent of the influence of the oil on the blend and the enzymatically interesterified products.

3.2. Materials and Methods

3.2.1. Materials

Refined, bleached and deodorized (RBD) PS and RBD PKO were purchased from Golden Jomalina Food Industries Sdn. Bhd., Malaysia. RBD FO was donated by Aarhus United, Aarhus, Denmark. Fatty acid compositions of the three oils are shown in Table 3.1. Lipozyme TL IM (*Thermomyces lanuginosa*), a silica granulated lipase donated by Novozymes A/S, Bagsvaerd, Denmark, was used for interesterification reaction in a

Table 3.1. Fatty acid composition of oils

Fatty acids	Area%		
	PKO	PS	FO
C6	0.5	n.d	n.d
C8	7.2	n.d	n.d
C10	6.3	n.d	n.d
C12	51.4	0.2	n.d
C14	16.3	1.2	5.7
C16	6.9	55.8	12.3
C16:1	n.d	n.d	7.8
C18	1.5	4.4	2.4
C18:1	8.2	29.9	17.6
C18:2	1.3	6.4	2.4
C18:3	n.d	0.3	12.7
C20:0	n.d	n.d	4.0
C20:1	n.d	n.d	8.8
C20:5	n.d	n.d	11.6
C22:6	n.d	n.d	14.7

Abbreviation: n.d.; not detected.

solvent-free system. GLC 51B and GLC 85 standards were purchased from Nu-Chek Prep Inc, Minnesota, USA. All chemicals and reagents for analysis were of analytical or chromatographic grades.

3.2.2. Methods

3.2.2.1. Triacylglycerol Analysis by Gas Chromatography

Samples were melted, dissolved in heptane (1% w/v) and transferred into 1.5 mL autosampler vials. TAG composition was determined by Hewlett Packard 5890 Series II Gas Chromatograph equipped with flame ionization detector and autosampler. Optima 1 column, 6 m X 0.32 mm i.d., 0.1 µm film thickness (Machery-Nagel GmbH and Co., Germany) was chosen for the analysis. The sample was introduced into the system by on-column injection technique. Flow rate of helium, the carrier gas, was at 8 mL/ min (measured at 90 °C). The initial oven temperature was 90 °C, held for 1 min, then increased to 260 °C at a rate of 30 °C /min. The oven temperature was further increased to 360 °C at 10 °C/ min and held for 5 min. Detector temperature was 360 °C whilst the injection port was set to oven tracking mode. The TAG composition was expressed as weight percent of the carbon number (CN), identified by comparing the respective retention times against the GLC 51 B standard.

Peak ratio (PR) between CN with major increment against major decrement was considered as the basis for calculating the interesterification degree (ID) of the interesterified blends (Zhang, 2004b). A PR of the highest difference TAG was used to monitor the interesterification degree which is defined as:

$$PR = \frac{\text{Area}\% \text{ of } (CN44 + CN46 + CN48)}{\text{Area}\% \text{ of } (CN36 + CN38 + CN50)}$$

All measurements were conducted in triplicates.

3.2.2.2. Fatty Acid Composition by Gas Chromatography

Fatty acid composition (FAC) was analyzed by HP 5890 Series II gas chromatograph. Lipids were methylated with sodium methoxide, separated by SP-2380 column (60m x 0.25 mm x 0.2 μ m) (Supelco, Bellefonte, PA, USA). Injector and detector temperatures were 260 and 280 °C respectively. The initial oven temperature was 70 °C, increased to 160 °C at a rate of 15 °C /min, then increased to 200 °C at 1.5 °C/min and held for 15 min, and finally to 225 °C at 30 °C/min and held for 10 min.

3.2.2.3. Solid Fat Content

SFC was measured according to AOCS Official method Cd 16b-93, using NMR spectrometer, Bruker Minispec mq series 2001. Serial measurements were conducted at 5, 20, 30, 35 and 40 °C, which were reported as the average of duplicate determinations.

3.2.2.4. Dropping Point

Dropping point (DP) analysis was conducted according to AOCS Official Method Cc 18-80, using Mettler Toledo FP 90 Central Processor and FP 83 HT Dropping Point Cell. Sample cup was kept in a freezer at -15 °C for 10 min, filled with sample and kept in the same freezer for another 15 min. Finally the cup was transferred to the analyzer. The initial furnace temperature was 38 °C and increased to the final temperature at 1 °C/min. Results were reported as mean values of triplicate determinations.

3.2.2.5. Experimental Design

A fractional factorial design based on response surface methodology with the assistance of MODDE 7 (Umetrics, Umeå, Sweden). Using three factors and three levels, 13 oil blends were generated and formulated. The factors were PS, PKO and FO as blend formulations within a defined range of 60-90, 10-40 and 0-10% by weight respectively. The effects of FO on flavor and odor have to be considered while defining the ranges of the oils. Most consumers will not accept a product that has fishy odor and taste, therefore, the maximum quantity of FO was limited to 10%. PK was more expensive than PS; therefore, a reasonable range was decided by adding 10 to 40% of PK in the blends. The responses were SFC (SFC5, 20, 30, 35 and 40 °C) and DP. Responses were fitted with the factors by multiple regression and the fit of the model was evaluated by coefficient of determination (R^2) and analysis of variance. The significance of the results was established at $P \leq 0.05$. Thirteen formulated blends (Table 3.2) were designed by response surface methodology, inclusive of triplicate blend of PS/PKO/FO 73/22/5% as the reference blend and duplicate of PS/PKO/FO 60/40/0%.

3.2.2.6. Enzymatic Interesterification

The above designed blends were subject to EIE in a continuous packed bed reactor. The reaction was conducted in an oven which was heated to 60 °C. A stainless steel column was filled with 12.8 g Lipozyme TL IM (*Thermomyces lanuginosa*) lipase and heated to 70 °C by a circulated water bath. The column dimension was 200 mm long and 15 mm i.d. and both ends were plugged with defatted cotton. Firstly, the column was flushed with PS for 2 h at a flow rate of 3 ml/min for water removal. Samples were collected after the column

Table 3.2. Experimental design: blend formulations and the responses before and after enzymatic interesterification (mean value of duplicate analyses for SFC and triplicate for DP).

Factors				Responses before EIE						Responses after EIE					
Exp. No	PS	PKO	FO	SFC (%)						SFC (%)					
				40°C	35°C	30°C	20°C	5°C	DP	40°C	35°C	30°C	20°C	5°C	DP
1	0.60	0.40	0.00	5.3	8.0	13.2	32.2	80.2	49,0	0.0	7.4	18.1	47.9	79.3	42.0
2	0.60	0.40	0.00	5.4	8.3	13.1	32.3	78.7	49,0	0.2	7.5	18.0	47.8	80.1	41.1
3	0.90	0.10	0.00	10.0	16.5	25.2	53.3	83.7	54,4	14.0	22.6	34.0	59.9	83.6	46.1
4	0.60	0.30	0.10	5.7	8.3	13.7	29.4	71.1	46,5	1.1	7.2	16.0	40.5	71.6	41.1
5	0.80	0.10	0.10	8.7	13.7	20.9	45.3	75.2	52,1	9.3	15.9	25.0	48.4	75.0	44.3
6	0.80	0.20	0.00	8.2	13.4	20.9	45.1	82.8	53,5	7.9	17.2	28.3	56.2	81.6	43.8
7	0.60	0.33	0.07	5.5	8.1	13.5	30.0	72.8	47,3	0.6	7.5	16.7	43.1	74.1	41.0
8	0.87	0.10	0.03	9.4	15.4	23.6	50.5	81.1	52,7	12.3	20.3	31.1	55.9	80.5	45.3
9	0.83	0.10	0.07	9.1	14.3	22.5	47.0	83.3	51,9	10.6	17.7	27.7	52.4	77.4	44.9
10	0.67	0.23	0.10	6.6	10.0	16.0	34.3	71.8	48,0	3.4	10.2	19.0	43.1	72.2	43.0
11	0.73	0.22	0.05	7.3	11.3	17.9	38.8	76.8	48,4	5.3	13.2	23.4	49.8	77.7	42.9
12	0.73	0.22	0.05	7.3	11.3	17.9	38.5	77.7	48,7	5.2	13.4	23.5	49.6	77.4	43.9
13	0.73	0.22	0.05	7.1	11.3	17.8	38.5	77.1	48,6	5.2	13.3	23.3	49.7	77.3	43.1

Average standard deviations for SFC and DP values are 0.5 and 0.3, respectively.

had been conditioned with three bed volumes of the substrate (PS/PKO/FO) to be used. Samples after such operation usually contain 1-2% FF, which was then reduced to less than 0.1% by short path distillation (KD4, UIC, Koln, Germany) (Zhang, 2005). The general conditions for the distillation were: evaporation temperature 150°C, condenser temperature 60°C, feeding tank temperature 60°C, vacuum 0.001 mbar, and flow rate 150 mL/h. The final products were stored at -40 °C before analyses.

Statistic Analysis

Data were analyzed by means of response surface methodology using commercial software Modde 7.0 from Umetrics (Umeå, Sweden). The response surface model was fitted to the following equation:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j \quad (1)$$

where Y is response variables, β_0 intercept, β_i first-order model coefficients, β_{ii} quadratic coefficients for the *i*th variable, β_{ij} interaction coefficients for the interaction of variables *i* and *j*, and X_i are independent variables. Responses were first fitted to the factors by multiple regression. The goodness of fit of the model was evaluated by the coefficients of determination (R^2) and the analysis of variances (ANOVA). The coefficients (95% confidence interval) were used for interpreting the influence of the factors, especially for fish oil, on two responses, i.e. SFC and DP.

The relationship between the physical and chemical properties was evaluated by principal component analysis with the assistance of Unscramble 9.1 (CAMO, Norway). A preliminary ANOVA partial least squares regression (APLSR) was performed on the results to project away differences in the assessors. All data were analyzed by APLSR using the three oils as X-data and the measured properties (solid fat content and triacylglycerol profile measured as carbon numbers) as Y-data.

3.3. Results and Discussion

3.3.1. Enzymatic interesterification

In order to avoid acyl migration during reaction, a packed bed reactor was used instead of a batch reactor (Xu, 2003). The different conversion degrees were controlled by adjusting the flow rate which presented by different residence time (Figure 3.1), so that, in reality, different interesterified products could be obtained. When the residence time of blends in the PBR was above 0.5 h, the reaction nearly reached the equilibrium. Therefore, this condition was used for all the enzymatically interesterified products.

The inclusion of PK and FO in the blends led to a broader range of triacylglycerol (TAG) composition from CN 30 to CN 58 (Figure 3.1). After enzymatic interesterification the composition of short (CN30-CN38) and long (CN50-CN56) chain TAGs were decreased while the intermediate (CN40-CN48) TAG chain numbers were increased. This indicates that rearrangement of the FAs in the TAG molecules have taken place. The decrement and increment of the TAG were the opposite of Ghosh and Bhattacharyya (1997) findings. In their work, high melting PS (melting point: 58.8 °C) was blended with liquid oils (either SFO, SBO or RBO) and subjected to batch EIE by *M. miehei* lipase. They reported that in all interesterified products, C48 and C52 glycerides decreased remarkably with a corresponding increased of C50 and C52 when compared to their blends. The blend contained 40% high melting PS and 60% liquid oil. Palmitic acids at the outer positions in the PS were replaced by unsaturated or other FAs from the liquid oils, thus reduced the composition of C48.

In this study, fish oil was added up to 10% of the total composition of the blend. During EIE, palmitic acids located at the outer positions of FO TAG were replaced by other FAs from PS and PKO and the cleaved palmitic acids were reattached to the free sites. This led to the increment of C48 and corresponding decrement of C50 and C52 as observed in this study. Even though there were no changes in the fatty acids composition, TAGs of the interesterified products have been changed due to the rearrangements of fatty acids in the glycerol backbone (Zhang, 2004b).

3.3.2. Model fitting

The formulation model was fitted based on data in Table 3.2 with the assistance of MODDE 7. SFC values measured at different temperatures and DP were taken as responses and blend ratios between the three oils were the variables. One of the major purposes of such modeling is to develop the mathematical connections between responses and variables so that future predictions can be made. In this case, the quadratic models were fitted without subjective refining with multiple regressions. There were not outliers of all the blends and measurements. The model coefficients for all the responses were statistically calculated and can be seen in Figures 3.2A1, 3.2B1, and 3.4. All P values of the coefficients were below 0.1. The coefficients of determination (R^2) of the models were also satisfactorily high (more than 0.95). According to the analysis of variances, there was no lack of fit and very satisfactory results were obtained. A few verification blends within the designed ranges were made and enzymatically interesterified. The measured SFC and DP values were nearly equal to those predicted (less than 5% variation). This indicates that the models well represent the actual relationships between physical properties and blend ratios.

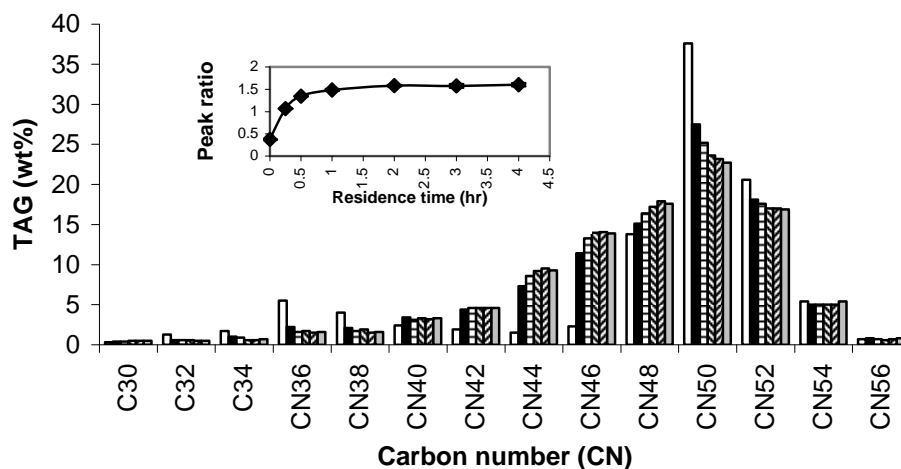


Figure 3.1. Effects of different residence time on triacylglycerol (TAG) profiles in a packed-bed reactor. Reaction conditions: PS/PK/FO (72.5/22.5/5, w/w/w), 12.8g Lipozyme TL IM, 70 °C, the residence time at 0 h, □; 0.25 h, ■; 0.5 h, ▨; 1 h, ▩; 2 h, ▪; 3 h, ▫ .

3.3.3. Effects of FO on SFC for the blends

Effects of FO and the interactions between the factors (PS, PK, and FO) on SFC were evaluated by SFC coefficients and their contour plots (Figures 3.2 and 3.3). For the pure blends, FO had a major impact on SFC at 5 °C (Figures 3.2A1 and A2). Addition up to 10% FO in the blends decreased SFC by 7.5-8.5 % (Figure 3.3A4). With the increase of SFC measurement temperature, the effect of FO was decreased. In general adding fish oil into the blends suppressed the increase of SFC (Figure 3.2A1). The interaction effects between the three factors were generally less significant compared to the individual components, indicating that the models are generally dominated by linear parameters.

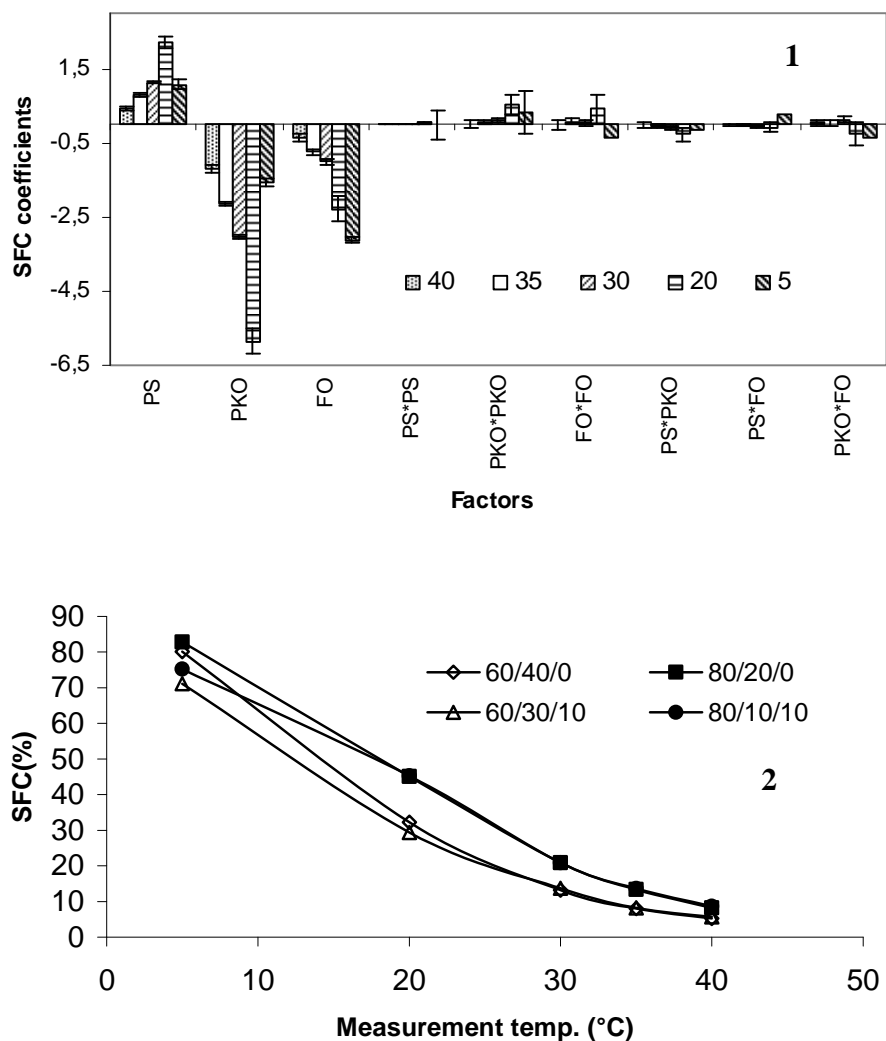


Fig. 3.2A. Effect of fish oil on solid fat content for the blends evaluated by factor coefficients from experimental design.

3.3.4. Effects of FO on SFC for the enzymatically interesterified products

For the enzymatically interesterified products, the impact of FO on SFC was expanded from 5 to 20 °C (Figures 3.2B1 and 3.2B2), which had 1.5*5 (7.5 °C) and 1.5*6 (9 °C) cross sections (Figures 3.3B4 and 3.3B3). These changes of physical properties were due to the re-arrangement of fatty acids on the glycerol backbones after enzymatic

interesterification. Such rearrangement caused the change of crystallization behavior (Zhang, 2005). The change of SFC profiles was visualized in Figure 3.2B2. Increase of PK content from 10 to 40wt% in the blends led to the decrease of SFC about 16, 15, and 14% at 30, 35 and 40 °C, respectively. Generally, the significant effect of second order parameters and interactions between PS, PK and FO were PK*PK (+) and PS*PK (-) at 40 °C, and PK*FO (+) from 30 to 40 °C (Figure 3.2B). FO effect (single factor) was insignificant at 40 °C.

Therefore, the positive interaction between FO*PK was pronounced at 40 °C than the other factors. This means that, by adding fish oil, the SFCs of interesterified products slightly increased. Table 3.2 shows that for the blends of PS/PK/FO (60/40/0 vs. 60/30/10 and 80/20/0 vs. 80/10/10) with adding 10% FO, SFCs at 40 °C were slightly increased by 1.0-1.6% compared to without adding fish oil for the interesterified products.

For the comparison of pure blends and interesterified products, we can see that after reactions, SFCs at 5 °C remained unchanged, but they were highly increased for SFC at 20 °C (Table 3.2). SFCs of EIE products had major changes at 20 and 30°C (not shown) compared to the blends, in which an increase of SFC was observed for PS, PK and FO within a defined ranges of 60-90 wt%, 10-40 wt% and 0-10 wt%, respectively. The degree of increasing SFC at 20 °C was reduced by adding fish oil into the blends compared to PK. SFC at 20 °C is an important parameter for product hardness during margarine packaging

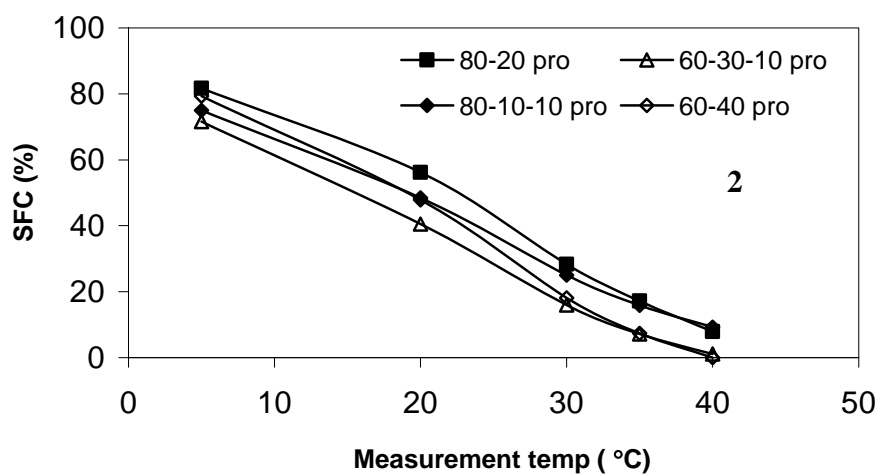
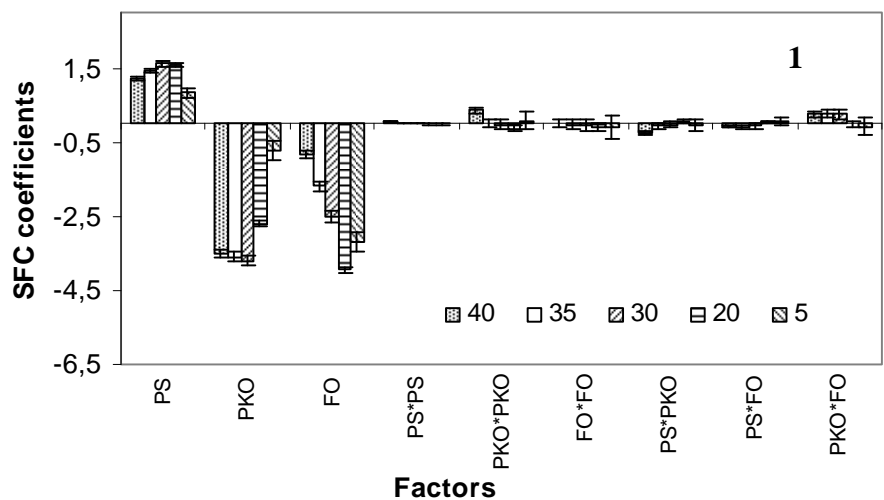


Figure 3.2B. Effect of fish oil on solid fat content for the enzymatically interesterified products evaluated by factor coefficients from experimental design.

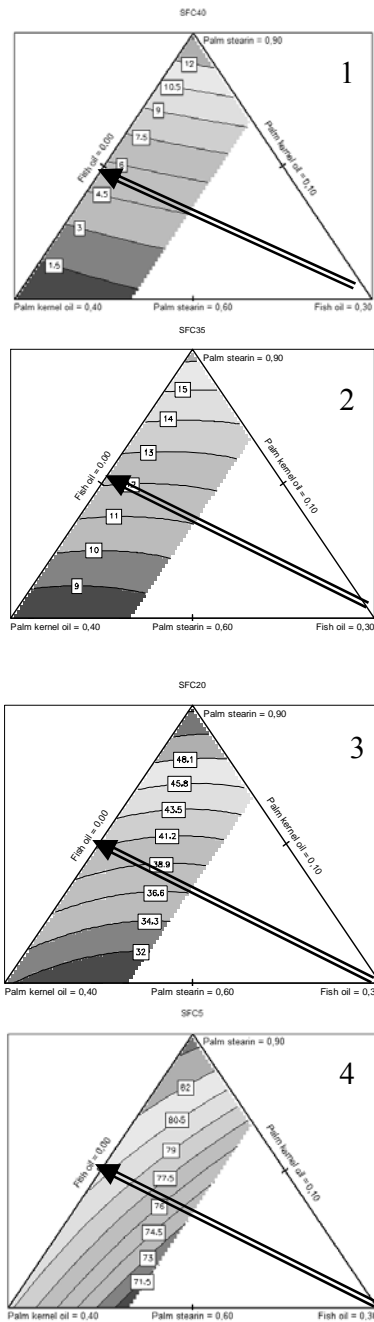


Figure 3.3A. Contour plots among three factors on SFC at measurement temperature 40, 35, 20 and 5 °C (from top to bottom) for the blends.

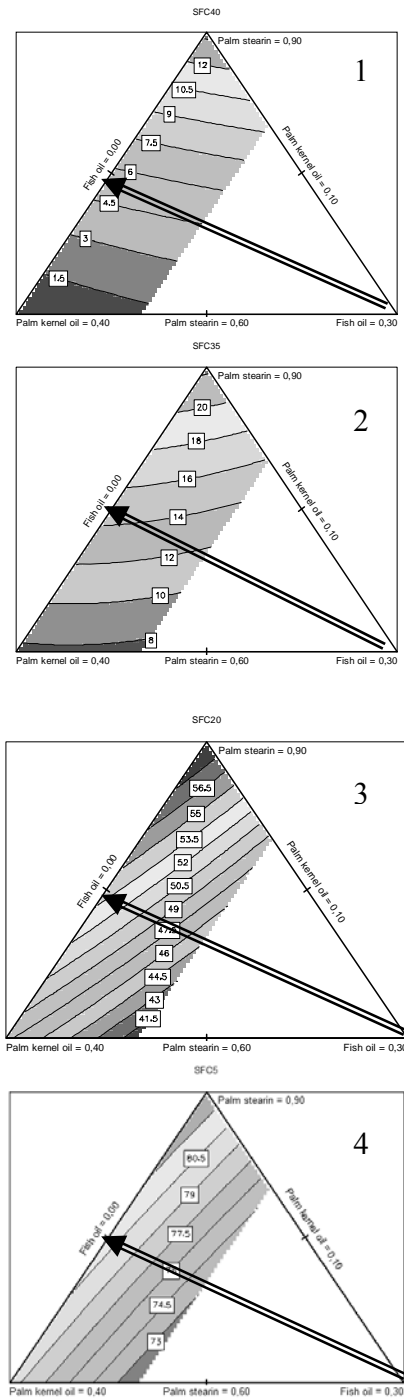


Figure 3.3B. Contour plots among three factors on SFC at measurement temperature 40, 35, 20 and 5 °C (from top to bottom) for the interesterified products.

and storage (Hoffmann, 1989). Therefore, when fish oil is added into the blends for margarine fat production, this large reduction of SFC at 20 °C has to be considered in order to produce stable products during storage.

For the interesterified products, SFC at 5 °C remained unchanged after the reaction. This was not quite in agreement with the previous study from Zainal and Yusoff (1999), in which the SFC curves were in parallel after enzymatic interesterification. One of the reasons could be due to the differences of the feedstocks. Certainly other differences such as different reactor types and enzymes as well as different operation procedures could also cause the difference.

Overall, the effect of FO was different before and after enzymatic interesterifications. Before interesterification, the special effect of adding FO on SFC was only observed at 5 °C. After interesterification, however, FO made the SFC curves more flat, where SFCs at 5 and 20 °C were lower and SFC at 40°C was a little higher for the interesterified products than the original blends. Such a phenomenon could imply that fish oil cannot be simply treated as just another liquid oil when making blend formulations. Its different fatty acid profile has made the solidification behavior different from other natural oil blends.

3.3.5. Effects of fish oil on DP before and after enzymatic interesterification

FO in the blends had a significant effect on dropping point (Figure 3.4). Adding more FO in the blends led to the decrease of DP. However, the effect of FO for interesterified products was statistically insignificant. DP of the blends and interesterified products were

within 46.5 to 54.4 and 41.1 to 46.1 °C, respectively (Table 3.2). Even though EIE had lowered the melting temperature, the blend formulation could be further improved to have an interesterified product that would melt closer to body temperature. After interesterification, DP was generally decreased in a range of 5 to 9.7 °C for those thirteen blends. This is in agreement with Zhang *et al.* (2005).

3.3.6. Relationship between SFC and TAG composition

The addition of fish oil has an impact on the physical properties for both the blends and the enzymatically interesterified products. This is of course related to the change of TAG profiles. A quantitative description can be made in Figure 3.5. For both the blends (A) and the interesterified products (B), fish oil (FO) clearly differs from other two oils in terms of effect (distance away from the two oils in the plots). FO has influence to the change of carbon numbers of C56, C58, C60, and C54 for the blends (A) and more of C56 and 58 for the interesterified products (B). However, FO has strong impact on SFC at 5°C for the blends, while after interesterification, the impact expanded to the SFC at 20°C. This agrees with the evaluation from above approaches. Generally, a small addition of fish oil (up to 10%) into the blends targeted for margarine production can have a significant influence on the physical properties of the formulated oil feedstocks. This impact can be even more pronounced after enzymatic interesterification through acyl exchange and rearrangement. The inherent reason is certainly related to the composition profile in fish oil. It is difficult to conclude, however, such changes can be strictly correlated to either EPA and DHA amount and their locations or those 20-22 saturated and monounsaturated fatty

acids and their locations in the glycerol backbone (Nascimento et al., 2004). The effects of such fatty acids on crystallization and crystal patterns will be also interesting to know.

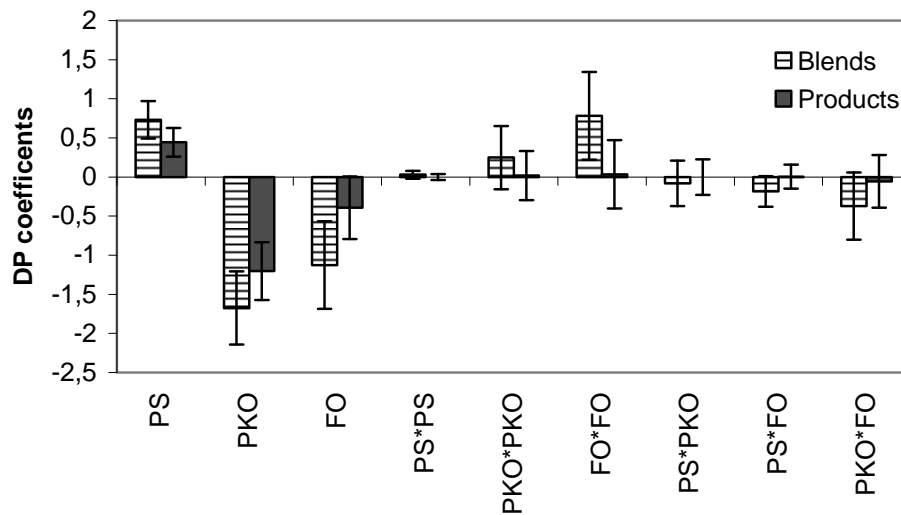


Figure 3.4. Effect of fish oil on dropping point for the blends and interesterified products evaluated by factor coefficients from experimental design. Mix: blend; pro: EIE product.

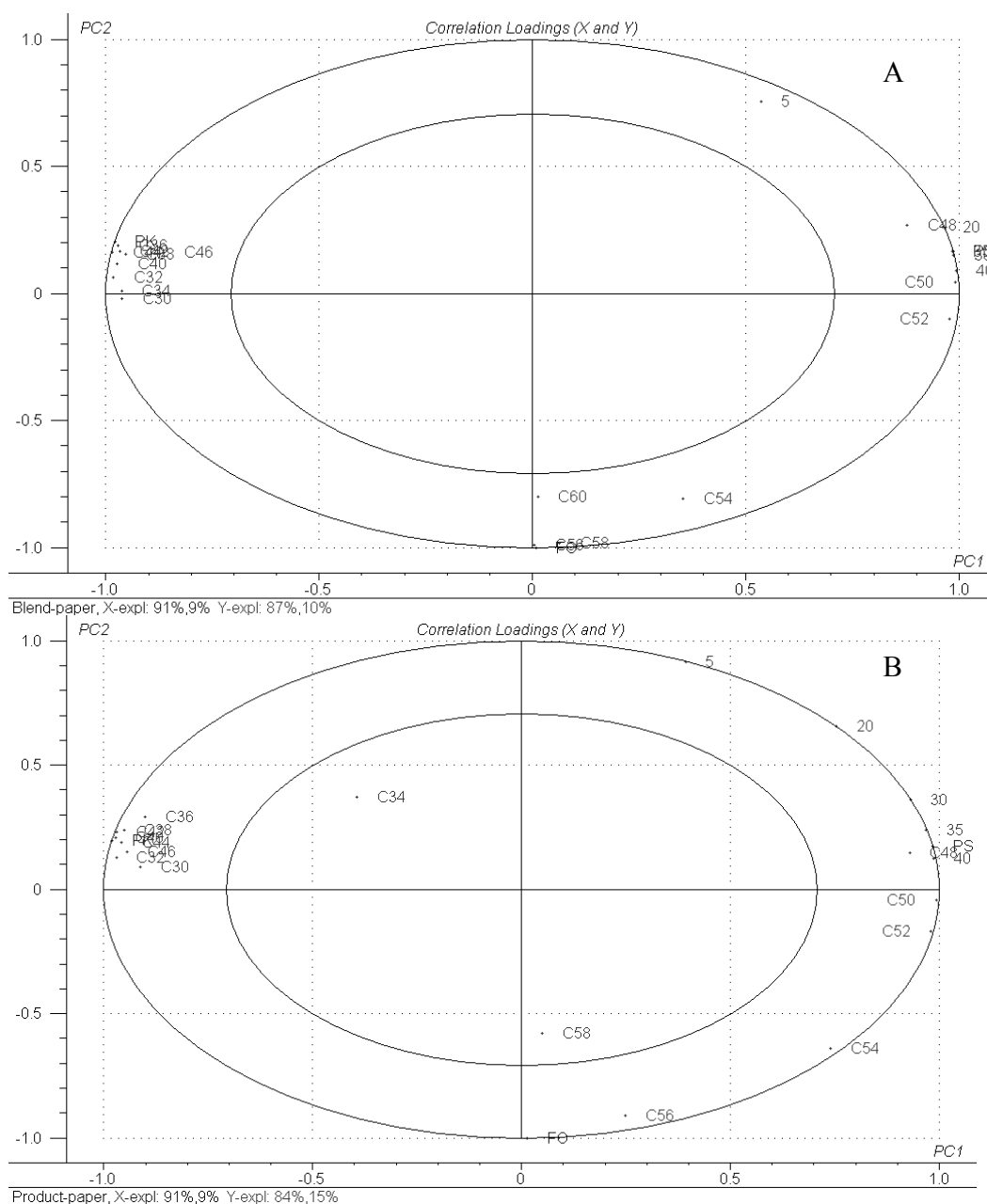


Figure 3.5. Correlation loadings plot of APLSR analysis on three oils as X-data and measured variables (SFC and carbon number of TAG from GC analysis) as Y-data. Numbers indicate SFC at the temperature ($^{\circ}\text{C}$). Numbers started with C indicate the carbon numbers of TAG. (A) Before EIE (pure blends) and (B) After EIE. The inner ellipse indicates 50 % explained variance and the outer ellipse 100 % explained variance.

3.4. Conclusion

Satisfactory quadric models were set up to study the effect of adding FO into a palm-based margarine formulation. Presence of 10% FO in the blend formulations comprising of PS (60-90 %) and PKO (10-40%) had negative effect on SFC profile. Presence of FO had different effect on the blend and the interesterified product. Before interesterification, the special effect of adding FO on SFC was only observed at 5 °C. After interesterification, however, FO made the SFC curves more flat, where SFCs at 5 and 20 °C were lower and SFC at 40°C was a little higher for the interesterified products than the original blends. Even though the amount of FO was only 10% in the blend formulation, yet it had a strong effect on SFC after EIE. Dropping point of EIE product was lower than the blend. However, the formulation could be further improved to get an interesterified product that melts at the body temperature.

Chapter 4

Enzymatic Interesterification of Palm Stearin and Coconut Oil by a Dual Lipase System

4.1. Introduction

The versatility of enzyme has paved the way for its wide industrial applications. Besides being a biodegradable compound, its selectivity, regiospecificity and mild operating conditions further added to the good traits. As ubiquitous enzymes, lipases (EC 3.1.1.3) constitute the most important group of biocatalysts for biotechnological applications (Jaeger & Eggert, 2002; Jaeger & Reetz, 1998). It is widely applied in organic chemistry, pharmaceuticals, cosmetics and leather processing, etc. (Schmid & Verger, 1998; Reetz, 2002). However, from industrial point of view, enzymatic modification of bulky oils and fats still represents one of the most promising technologies, in terms of processing scale and practical applications (Xu, 2000; Gunstone, 1998). As one of a few commercialized approaches, enzymatic interesterification has been intensively studied (Marangoni & Rousseau, 1995).

During the early days of its discovery, a lot of studies had been focussing on the development of new enzymes looking for new applications. The investigations cover enzyme evaluation, protocol development, fatty acid specificity, reaction optimization and packed bed reactions. These concerted efforts do provide a lot of useful information for better understanding the interaction among lipase, substrates and operation parameters, and the development of practical techniques (Gunstone, 1999). However, either in batch reaction or continuous operation single lipase is generally employed as biocatalyst. Little

attention has been given on dual or multiple lipase systems to reveal their synergistic or antergic effects (Cho et al., 2001).

Generally speaking, multiple enzyme systems have been assigned to successive reactions (Baisch, 1998; Adamczyk et al., 1997; Wang et al., 1993) or assistant actions to the main reactions by removing byproducts or enzyme pre-treatment (Sheu et al., 2001; Fujii et al., 1988). The former approach, also called combinatorial biocatalysis, can obviate some separation steps and simplify purification procedures. The latter operation could deplete the inhibiting byproducts of the enzyme for main reaction, which facilitate the accumulation of desired products. These investigations are not only scientifically interesting, but also result in significantly higher enzymatic turnout than that of the enzyme employed alone (Sheu et al., 2001). This concept has been applied to crude enzyme systems (consisting of multiple enzymes), by which Barthomeuf and Pourrat (Barthomeuf & Pourrat, 1995) showed a better performance for the production of high-content fructo-pligosaccharides using crude fructosyl transferase from *Penicillium* containing glycosidase. The combining utilization of non-specific and 1,3-specific lipases has been widely used for structured lipid production (Negishi et al., 2003). However, these consecutive syntheses usually were performed in two steps, accompanying the purification of intermediate products, in which the two types of lipases acted as biocatalyst separately. Obviously, this differs conceptually from a dual enzyme system, in which two biocatalysts simultaneously occurred, with possible interactions. A single step approach was reported by Hirose et al. (2006) where blended lipases was applied in synthesizing TAG containing conjugated linoleic acid. The lipases were *Alcaligenes* sp., *Penicillium camembertii* and *Rhizomucor miehei*. Combinations of

two lipases were reported to increase the incorporation of conjugated linoleic acid in the TAG.

The primary aim of this work is to have a close look on the possible interaction of two simultaneously occurring lipases in a dual lipase-mediated interesterification, and explore the possibility to achieve an improved reaction. As a typical system for margarine fat study (Zhang et al., 2001), palm stearin (PS) has relatively simple fatty acid composition (oleic + palmitic > 85%) and CnO enriches in medium-chain fatty acids (Table 4.1). Their blend shows concentrated distribution of triglyceride (TG) profiles (Figure 4.1A) and therefore was chosen as a model system in this study. 1,3-Specific lipases from different sources, existed in either free (Lipase from *Pseudomonas flourescens*) or immobilized form (Lipases from *Thermomyces lanuginosa*, *Rhizomucor miehei* and *Candida antarctica* B), were employed as biocatalysts for enzymatic interesterification. The synergistic effects of dual enzymes were evaluated by the comparison of the experimental observation of sole and two-mixed lipase reaction systems. This work attempts to present a new observation and consideration on the enzymatic interesterification mediated by a dual lipase system.

4.2 Materials and Methods

4.2.1. Materials

Palm stearin and CnO were purchased from Karlshamn AB, Karlshamns, Sweden. Lipozyme TL IM (*Thermomyces lanuginosa*), Lipozyme RM IM (*Rhizomucor miehei*), and Novozym 435 (*Candida antarctica* B) lipases were kindly provided by Novozymes A/S, Bagsvaerd, Denmark, while Lipase AK Amano 20 (*Pseudomonas flourescens*) was donated

by Amano, Nagoya, Japan. Fatty acid methyl esters used as standards were procured from Sigma Chemical Co. (St. Louis, MO). Tricaprin, trilaurin, trimyristin, tripalmitin, and tristearin used as the references for ECN determination were from Nu-Chek Prep, Inc. (Elysian, MN) and with minimum purity of 99%.

Table 4.1. Fatty acid composition of palm stearin and CnO oil (wt%)^a and blend oil (PS/CnO 7/3, w/w).

Fatty acid	Palm stearin	CnO oil	Blend oil (mol%)	Blend oil (wt%) ^b
Caprylic	0.07	8.02	4.16	2.46
Capric	0.00	5.88	2.50	1.76
Lauric	0.48	47.00	17.59	14.44
Myristic	1.29	18.39	6.87	6.42
Palmitic	52.38	9.00	37.89	39.37
Stearic	4.94	2.80	3.72	4.30
Oleic	33.44	7.01	22.28	25.51
Linoleic	7.19	0.12	4.46	5.07
Linolenic	0.20	1.78	0.59	0.67

^a Means of triplicate determinations. ^b The fatty acid compositions of blend oil were calculated by mass balance based on the average molecular weight of palm stearin and CnO oil.

4.2.2. Methods

4.2.2.1. Enzymatic Interesterification

Palm stearin/ coconut oil blend (7/3 w/w) was enzymatically interesterified by mixtures of enzymes. The enzyme types and the composition ratios are shown in Table 4.2, while Table 4.3 shows the enzymes' characteristics. Reactions were conducted in triplicate, carried out in 60 mL brown bottle placed in a water bath which was heated to 60 °C. The mixture in the bottle was stirred by magnetic stirrer throughout the reaction. The enzyme amount was 9% w/w of the substrate. Samples were withdrawn after 15 min, 30 min, 45 min, 1 h, 2 h and 3 h reaction times. 20 µL of aliquots were withdrawn at desired intervals and dissolved in 1 mL of hexane assigned for HPLC analysis.

To evaluate the effects of the carriers of immobilized lipases on enzyme activity, thermal inactivation of Lipozyme TL IM, Lipozyme RM IM and Novozym 435 were carried out in a vacuum oven. The immobilized lipases were kept at 160 °C for 24h under vacuum. The resulting preparations were kept at room temperature for 2h to partly recover lost moisture. The inactivated lipases were employed for interesterification of PS and CnO under the same condition as used for active lipase to assay the residual activity. After 16 h no detectable reaction was observed for all 3 inactivated lipases, indicating the inactivated immobilized lipases could be treated as a carrier. The resulting preparations were therefore used as a replacement of unavailable blank carrier for evaluation.

Lipozyme TL IM was used as a model to examine the effect of carrier property on the immobilized lipase-catalyzed reaction. Equal amount of inactivated preparation and

Lipozyme TL IM were mixed for enzymatic interesterification and compared with sole Lipozyme TL IM- catalyzed reaction. The same dosage of Lipozyme TL IM for all tests was used for comparison.

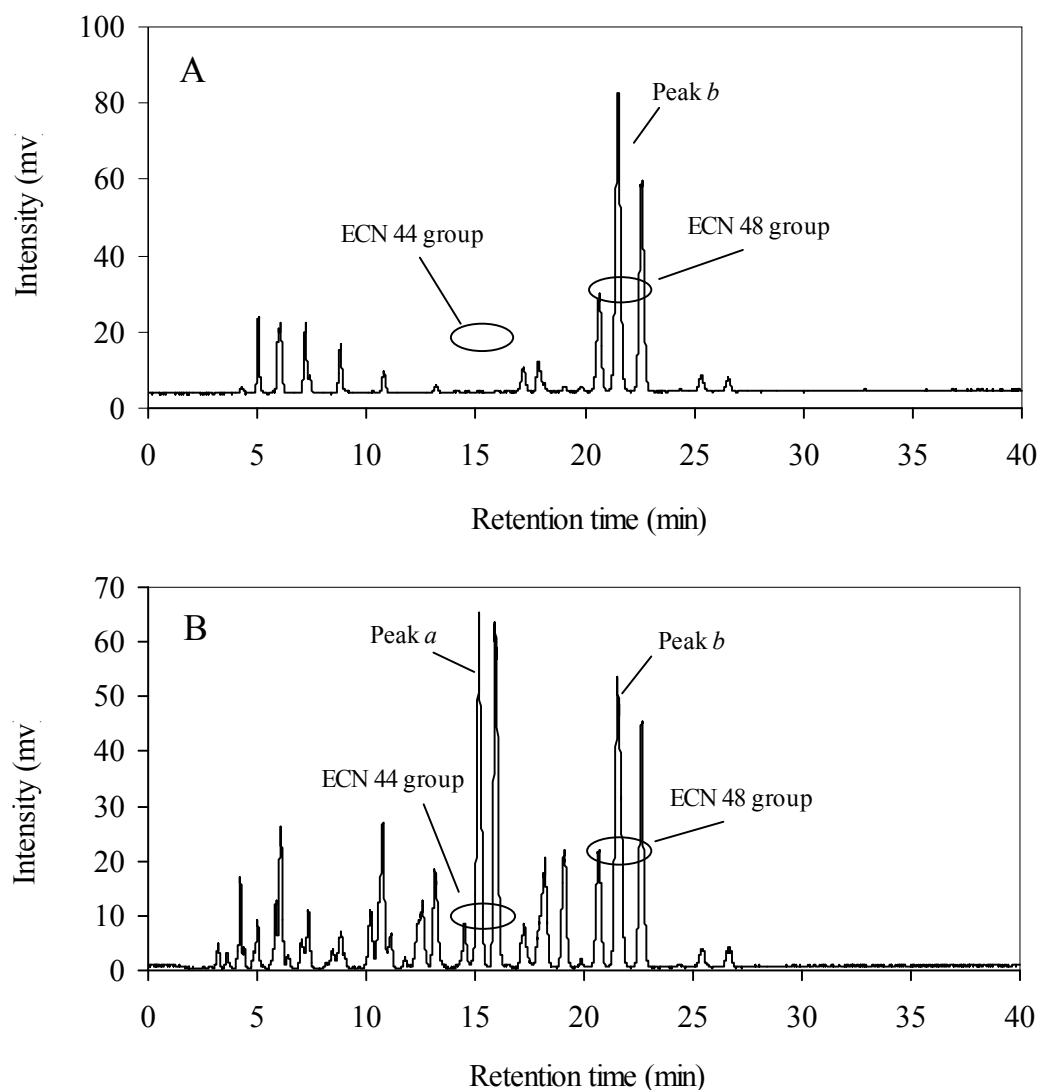


Figure 4.1 HPLC chromatograms of the triglyceride profiles of PS / CnO oil (7:3) blend before (A) and after 5h EIE (B) catalyzed by a dual lipase system consisting of 70% (wt%) Lipozyme TL IM and 30% Lipozyme RM IM. The changes of relative contents of central peak of ECN (equivalent carbon number) 44 (Peak a) and 48 (Peak b) groups with reaction evolution, which also represent the corresponding major component of representative group, were employed as an index to monitor reaction progress.

Table 4.2. Enzyme mixture and composition for batch reaction.

Lipase	Composition
AK Amano 20/ Lipozyme 435	
AK Amano 20/ Lipozyme TL IM	
AK Amano 20/ Lipozyme RM IM	Composition of each mixture
Lipozyme 435/ Lipozyme TL IM	was 70/30, 50/50 and 30/70
Lipozyme 435/ Lipozyme RM IM	
Lipozyme TL IM/ Lipozyme RM IM	

Table 4.3. Characteristics of the applied lipases

Lipase	sn-1, 3 regiospecificity	Immobilization
Lipozyme TL IM ^a	Yes	Yes
Lipozyme RM IM ^a	Yes	Yes
Novozym 435 ^a	Yes/ no (depend on reactant)	Yes
AK Amano 20 ^b	Slightly	No

Source: ^a Product sheet, Novozymes, Denmark

^b Xu (2000)

The effects of the carriers of the three immobilized lipases on the blended free enzyme were evaluated by comparing Lipase AK-inactivated preparation blend-catalyzed

interesterification with the reaction catalyzed by the same dosage of Lipase AK without inactivated immobilized lipase addition.

All reactions were performed in triplicate, and the means were used for result evaluation.

4.2.2.2. Determination of Fatty acid and Triglyceride Compositions

Palm stearin and CnO oil were methylated with Boron trifluoride-methanol method (Zhang et al., 2000). Fatty acid methyl esters were analyzed on a Hewlett-Packard 5830A GC system equipped with a 25 m fused silica capillary column (25QC2/BPX/0.25 μ m film, I.D. 0.22 mm) (Scientific Glass Engineering, Melbourne, Australia) and a flame-ionization detector (FID), as well as a HP 7671A autosampler. The injection temperature was set at 250 °C and helium was employed as carrier gas at a flow rate of 40 mL/min. Oven temperature programming was as follows: started from 70°C and held for 2 min; the temperature was increased to 210°C at a rate of 10 °C /min and maintained at 210°C for 5 min; and followed the second increase with the rate of 40 °C/min to 250 °C and held at 250°C for another 2 min. Fatty acid methyl ester peaks were identified by the comparison of retention time with standards.

Triglyceride composition of materials and products were determined with Hitachi-Merck HPLC Series 7000 (Hitachi-Merck, Japan), conjugated with a PL-ELS 2100 evaporative light scattering detector (ELSD) (Polymer Laboratories, Shropshire, UK). The reverse phase column employed was Supelcosil LC-18 (250 mm \times 4.6 mm) (Supelcosil Inc., Bellefonte, PA). The ELSD was operated at an evaporating temperature of 70 °C and a

nebulizing temperature of 50 °C with air as the nebulizing gas. Acetone and acetonitrile acted as the mobile phases by a gradient elution, beginning with an equal amount of the two solvents (50/50) and ending with 70% acetone and 30% acetonitrile. The mobile phase flow rate was 1.5 mL/ min. The TAG peaks were identified by comparing the retention time with authentic triglyceride standards. Area percentages were used as weight to quantify the triglyceride composition. All measurements were conducted in triplicates.

4.2.2.3. Evaluation Setup for Interesterification Extent and Synergistic Effect

The TAGs of oils and fats in HPLC chromatogram could be in general classified by equivalent carbon number (ECN). ECN depends mainly on carbon number and unsaturated degree of three bound fatty acids, of which the value equals to the total carbon number of acyl groups subtracted by the number of double bonds. The triglycerides with the same ECN could be eluted as adjacent but separate peaks by HPLC, forming so-called ECN group of TGs in chromatogram (Figure 4.1). For example, OOP, POP and PPP can be eluted as a group of peaks of ECN 48 in the order as shown in Figure 4.1.

As shown in Figure 4.1A, the mixture of PS and CnO oil exhibits characteristic TAG profiles, namely, the TAGs with medium chain length fatty acids located in the retention time range of 5 – 10 min and the ECN 48 group of TAGs. According to individual analysis HPLC analysis for PS and CnO, the former dominated the TAGs from CnO oil, while dominant ECN 48 group represents characteristic peaks of PS (Zhang et al., 2000). Interesterification leads to the rearrangement of acyl groups within or inter-triglycerides, resulting in the changes of relative contents of TAG profiles or the generation of new

triglycerides (Xu et al., 2006). The comparison of Figure 4.1A and 4.1B revealed that a significant change before and after reaction is the appearance of ECN 44 group of triglycerides (almost undetectable in the starting materials) and evident decrease of the relative contents of ECN 48 group of TAGs. Furthermore, these two groups of TAGs always occupy the major mass portion (>55%) during the reaction evolution. Thus, the change of relative contents of the two groups could be acted as an index to denote the reaction evolution. Representatively, Peaks a and b are the major component belonging to respective groups. Therefore, the interesterification degree (ID) can be simplified as:

$$ID = \frac{\text{area of Peak a}}{\text{area of Peak b}} \quad (1)$$

To quantitatively evaluate the reaction performance of a dual lipase system, herein we defined the Synergistic Effect Coefficient (SEC) as:

$$SEC (\%) = \frac{ID_{A \cdot B} - (x_A \cdot ID_A + x_B \cdot ID_B)}{x_A \cdot ID_A + x_B \cdot ID_B} \times 100\% \quad (2)$$

where ID_A and ID_B denote the individual interesterification degree of the reaction when Lipase A and B solely act as biocatalyst. $ID_{A \cdot B}$ is the corresponding reaction degree catalyzed by the enzyme mixture of lipase A and B with the mass fraction of x_A and x_B . For comparison, the total enzyme load of dual lipases is always the same as the amount of single lipase in this work. Clearly, the value of SEC can be positive, if the combination effect of two lipases is augmented; thereby, the value can also be negative, if the effect is diminishing. Therefore, this definition can be used to characterize a dual enzyme system qualitatively and quantitatively.

4.3. Results and Discussion

4.3.1. Synergistic Effects of Lipozyme TL IM and RM IM

Figure 4.2 presented the time courses of interesterification of PS and CnO catalyzed by sole Lipozyme TL IM and RM IM and their mixture at different proportions. Compared with Lipozyme RM IM, Lipozyme TL IM achieved a better initial rate or a higher final interesterification degree after 24h, which agreed with the previous observation that Lipozyme TL IM shows a better performance for interesterification (Rønne et al., 2005a). Before 4h, the interesterification degree of dual lipases catalyzed reaction ranked between Lipozyme TL IM- and RM IM- mediated reactions, and at the identical time the ID decrease with the increase of RM IM proportion in the two lipase mixtures. Provided that the two lipases act separately with little interaction, this observation is reasonable because the introduction of RM IM to TL IM should result in a lower total reaction rate. Interestingly after 6h, dual enzyme systems, especially for the mixture of 70% TL IM and 30% RM IM, obtains a higher reaction degree (0.93 at 24h) than the sole Lipozyme TL IM system (0.89 at 24h).

To more accurately evaluate the synergistic effects of dual lipase systems, theoretical ID (calculated from individual IDs of the two lipases and their propositions in the mixture) as well as SEC of two enzyme mixtures with different ratios are depicted in Figure 4.3. It is clear that for the mixtures of Lipozyme TL IM and RM IM at any test ratios the experimental interesterification degrees are higher than the corresponding theoretical values. The strength of the synergistic effects is also shown to be associated with the ratio of two mixed lipases (Figure 4.3). The SEC values of TL IM – RM IM (60:40) varied in

the range of 3 – 5 during the time course and TL IM – RM IM (50:50) around 5.5-11. While TL IM – RM IM (70:30) at 2h exhibits 10% reaction amelioration over the expected value. However, with the reaction progress this synergism comes to closer as indicated in Figure 4.3. Overall, a synergistic effect between two immobilized lipases from *Thermomyces lanuginose* and *Rhizomucor miehei* seems to be operative, even though these positive impacts are not very significant. The real reason accounting for the synergism of dual lipase systems is not fully clear. Although two enzymes co-exist in the same medium environment, the microenvironment for each lipase is not the same. Even though we neglect the non-specific effects of lipases (Cao et al., 2003), the nature of carrier, fatty acid selectivity of lipase and the immediate interaction between the two lipases bound to two different particles can influence the overall reaction performance of a dual immobilized lipase system (Guo & Sun, 2004).

4.3.2. Interaction Between Lipases

To get further understanding on the synergistic effect of a dual immobilized lipase system, we conducted similar reactions employing the dual enzyme combinations of Lipozyme TL IM – Novozym 435, and Lipozyme RM IM – Novozym 435 with different mixing ratios (Table 4.4). The results shown in Table 4.4 demonstrate that a positive interaction between two mixed lipases does not always happen. The results also indicate that the interactive effect differs from different lipase combinations and also depends on the mixing ratio. The negative SEC values of Lipozyme TL IM – Novozym 435 at the ratio of 7/3 and 3/7 at the early stage of the reaction demonstrate an antergic effect possibly occurred in this dual lipase system at the test ratios.

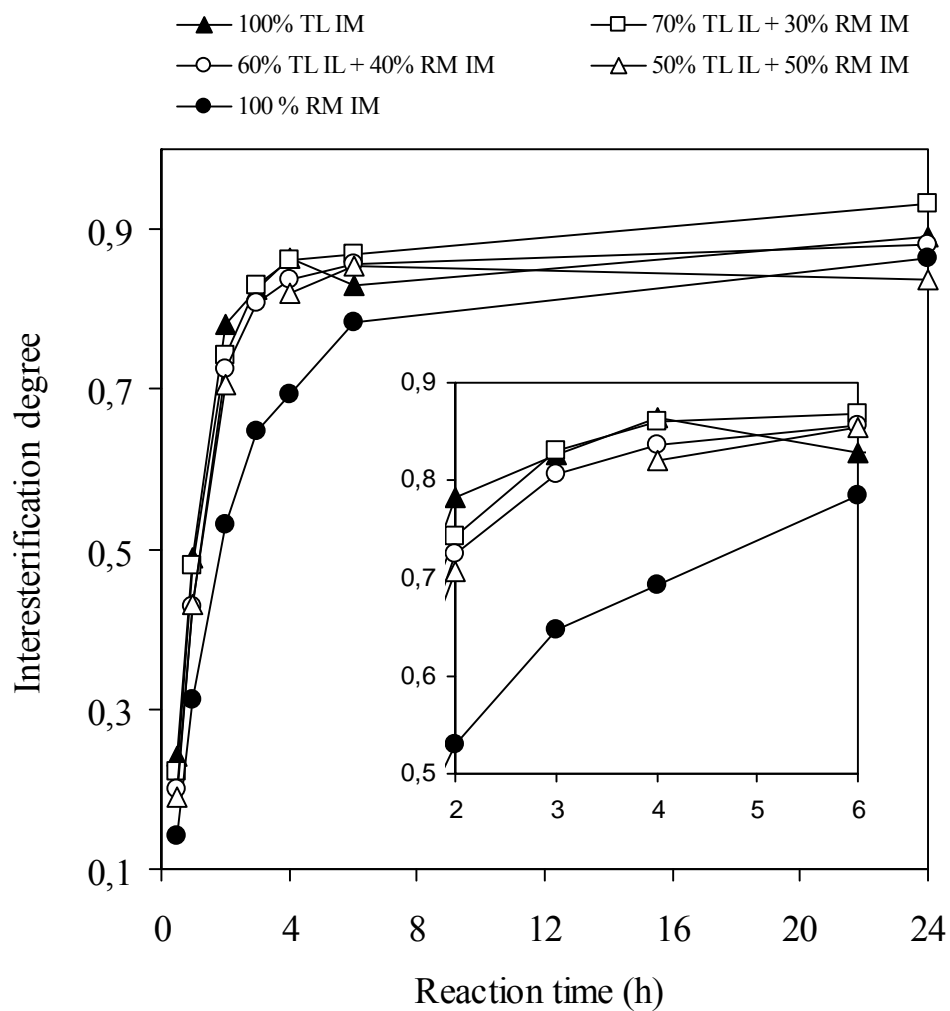


Figure 4.2 Time course of enzymatic interesterification catalyzed by Lipozyme TL IM, Lipozyme RM IM, or their mixture with different proportion. The inserted figure is an enlarged part of the time range of 2 – 6 h. The batch reactions were conducted at 60 °C with magnetic agitation in triplicate. The enzyme load either for sole or dual lipase system is 9% (wt%) of substrate.

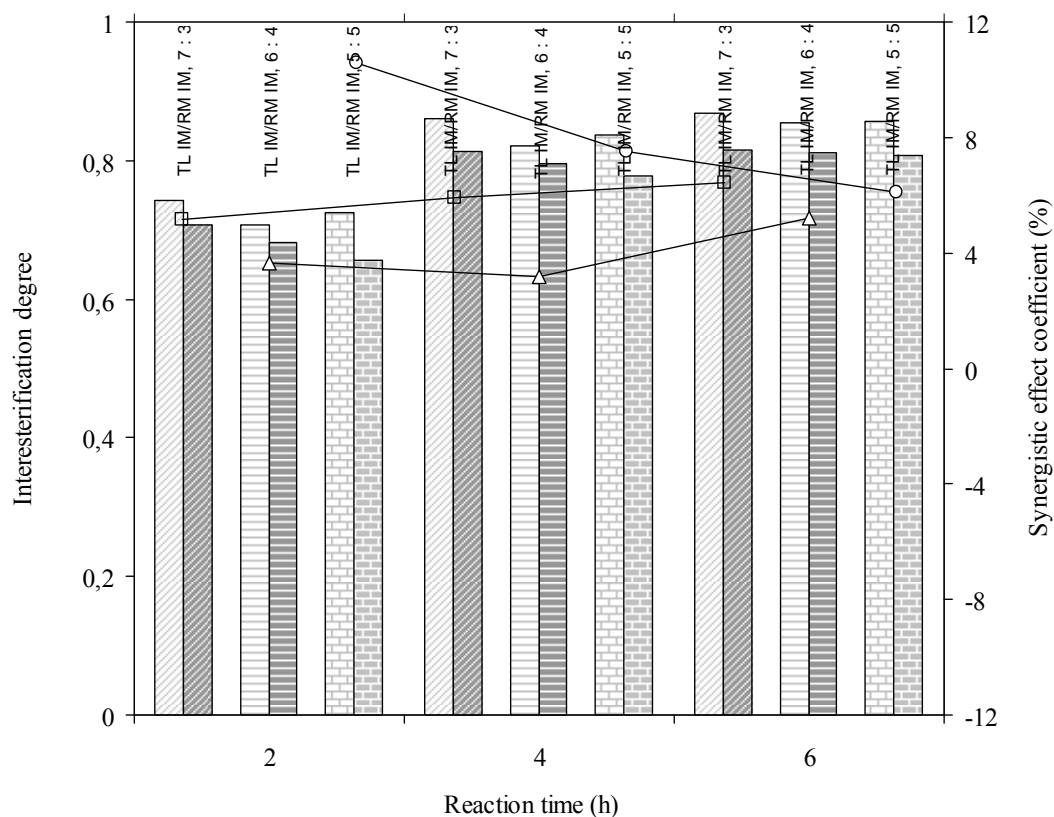


Figure 4.3 Synergistic effects of dual enzyme systems of Lipozyme TL IM and RM IM mixed with different ratio (TL IM / RM IM). The reaction condition is the same as in Figure 4.2. The bars with light color represent experimental interesterification degree and those with dark color are the corresponding theoretical values calculated by the weighted ID sum of two combined lipase when they catalyze interesterification solely. Synergistic effect coefficients (\square , \diamond and \circ) were calculated with Eq. (2).

Similar phenomena have been observed for another dual lipase system of Lipozyme RM IM – Novozym 435 at the mixing ratio of 7/3. Interestingly, the dependency of the interactive effects on the mixing ratio of two lipases is experimentally repeatable. The real

reasons accounting for above observations are not clear. However, our results seem to suggest that the antergic interaction existed among two mixed immobilized lipases might be related to the nature of the carriers of the immobilized enzymes. Because, as the corresponding carrier of Lipozyme TL IM, RM IM and Novozym 435, granulated silica, hydrophobic resin, and polyacrylate beads have different hydrophobic, hydrophilic and electrostatic properties. The interaction among the immobilized particles might influence the aggregation or dispersion in the reaction mixture, and accordingly affect the apparent activity of the enzyme mixture. However, with the reaction progress, the carrier particles could be totally soaked by oil and the porous matrices of particle is filled with oil molecules to isolate the particles from each other. This probably contributes to the attenuation and disappearance of the antergic effects in the later stage (Table 4.4).

It is worthy to note that a significantly synergistic effect on enzymatic interesterification during the whole time course occurred, when equal amount of Lipozyme TL IM – Novozym 435 or Lipozyme RM IM – Novozym 435 mixture employed as biocatalyst. In comparison with the corresponding single enzyme system, the dual lipase system not only showed a faster reaction, but also achieved a higher interesterification degree (Table 4.4). The reason why the dual lipase system at this mixing ratio other than lower/higher proportion exhibited synergism remained to be explored. However, the higher ID is most likely related to the fatty acid selectivity of lipases and the composition of substrates used in this study.

Table 4.4 Synergistic effects of enzymatic interesterification of palm stearin and CnO oil catalyzed by dual lipase systems.

	Ratio of two lipases	Lipozyme TL IM : Novozym 435					Lipozyme RM IM- Novozym 435			
		10:0	7/3	5:5	3:7	0:10	10:0	7:3	5:5	3:7
2 h	^a ID	0.7819	0.3388	0.7820	0.4912	0.4675	0.5306	0.4143	0.5691	0.4760
	^b SEC (%)	-	-50.73	24.39	-12.57	-	-	-18.96	14.10	-2.12
4 h	^a ID	0.8291	0.6557	0.8763	0.7263	0.6967	0.7847	0.6979	0.8398	0.8797
	^b SEC (%)	-	-26.10	14.85	-1.40	-	-	-3.06	18.29	24.50
8 h	^a ID	0.8311	0.8826	0.9318	0.8567	0.9023	0.8012	0.8788	0.9277	0.9543
	^b SEC (%)	-	3.54	7.51	-2.75	-	-	5.64	8.92	9.46

^a Means of triple batch experiments. 9% of substrate was used as the dosage of biocatalyst for either single or dual lipase system and all other conducting conditions were identical. ^b Synergistic effect coefficient (SEC) was calculated by Eq. (2).

Pleiss et al. (1998) has demonstrated the molecular basis of substrate specificity, revealing that the fatty acid selectivity of lipases is dependent on the shape and physico-chemical properties of the scissile fatty acid binding site of lipases. *Rhizomucor miehei* lipase (Lipozyme RM IM) with a hydrophobic, crevice-like binding site located near the protein surface, has a relatively low activity for short-chain fatty acids, but increasing activity for longer fatty acid. *Candida antarctica* lipase B (Novozym 435) with a funnel-like binding site has high activity for short and medium chain length fatty acids (Rangheard et al., 1989). Rønne et al. (2005a) showed that *Thermomyces lanuginosa* lipase (Lipozyme TL IM) is non-selective towards fatty acids. As indicated in Table 4.1, there exist considerable

proportion of short or medium chain fatty acids in CnO oil and the blend. LaOP and LaPP (La, lauric acid) constitute the major components of ECN 44 group of triglycerides, which was used to monitor the reaction (Figure 4.1). These facts mean that the enzymatic interesterification of PS and CnO involves substantial rearrangement of short and medium chain length fatty acids. It is therefore pronounced that, in a dual enzyme system, the lipase with high activity for short and medium chain fatty acids acted in concert with the lipase favoring longer chain fatty acid could result in an improved overall reaction rate and an enhanced interesterification degree.

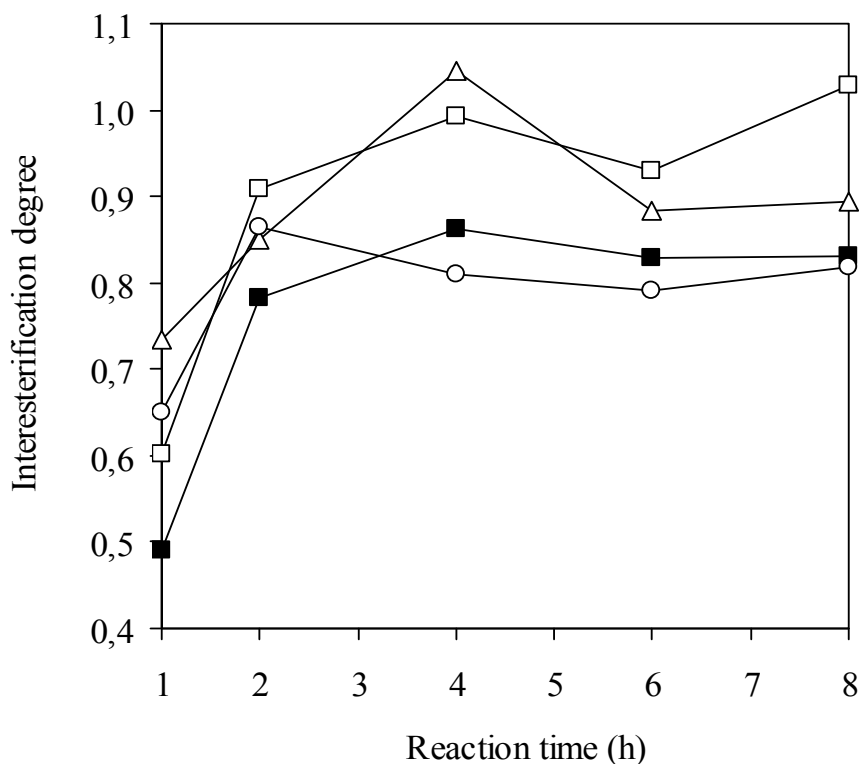


Figure 4.4 Effects of the carriers of immobilized lipases on Lipozyme TL IM catalyzed interesterification. The biocatalyst consists of 9% (wt% of substrate) Lipozyme TL IM and the same amount of inactivated Lipozyme TL IM (□), RM IM (Δ) and Novozym 435 (○). The Lipozyme TL IM without inactivated immobilized lipase (■) was used as a control.

4.3.3. Effects of Carriers on Lipozyme TL IM-Catalyzed Interesterification

The effects of the carriers of three immobilized lipases (the inactivated preparations) on Lipozyme TL IM-catalyzed interesterification have been depicted in Figure 4.4. Compared with sole Lipozyme TL IM-catalyzed interesterification, the contributions of co-existed inactivated Lipozyme TL IM and RM IM to the enhancement of the reaction were significant at any monitoring time. Inactivated Lipozyme TL IM generally produced over 15% ID addition at the identical reaction time; and the inactivated Lipozyme RM IM gave 50% enhancement of ID at 1h and 21% increase at 4h, respectively. However, the inactivated Novozym 435 showed a different behavior, i.e. a positive contribution before 2 h and a negative effect at 4 h and 6 h as well as a close ID with single Lipozyme TL IM at 8 h.

It should be pointed out that, after inactivation process at high temperature, the structure of the protein of Lipozyme TL IM could be changed, as the color became brown. The resulting preparation showed good mixing with Lipozyme TL IM, behaving as a hydrophobic carrier. Contrarily, the structure of the carrier of Novozym 435 seems to change little (No color change observed). Aggregation was observed when mixed with Lipozyme TL IM, showing more hydrophilic property. No apparent change for inactivated Lipozyme RM IM can be seen (due to original brown color) and a good mixing with Lipozyme TL IM was also observed. These observations not only explain the results in Figure 4.4 but also support our assumption that the dispersion state of immobilized lipases may affect dual lipase-catalyzed reactions. Hydrophobic carrier could be well dispersed in oil blend and help the dispersion of the co-existed lipase, while hydrophilic carrier is not

good for dispersion in hydrophobic reaction mixture and also induce the aggregation of co-existed immobilized lipase. This might also help to understand why the synergistic effect could always be observed for the Lipozyme TL IM – RM IM dual system (Figure 4.3) but not always for the Lipozyme TL IM – Novozym 435 system (Table 4.2), if one considers hydrophobic property of RM IM carrier differing from the carrier of Novozym 435.

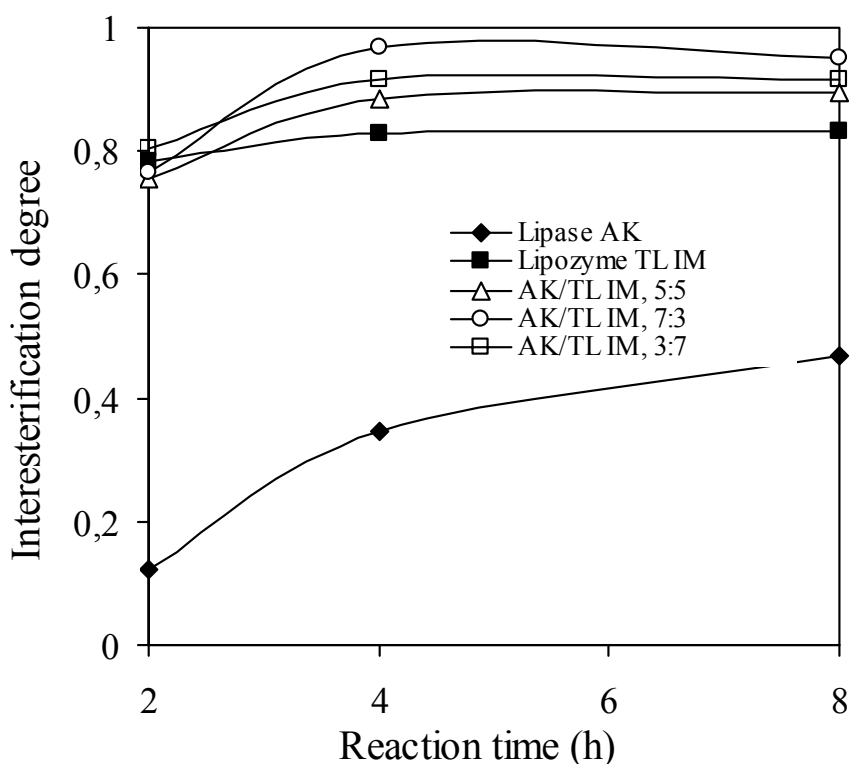


Figure 4.5 Synergistic effects of free (Lipase AK) and immobilized (Lipozyme TL IM) lipases. The total amount of free or immobilized enzyme as well as their mixtures were 9% (wt%) of substrate.

4.3.4. Dual Enzyme System of Free and Immobilized Lipases

To examine the effects of different occurring form of enzymes in a combined system, Lipase AK Amano 20 (powder) was mixed with Lipozyme TL IM at the ratio of 7/3, 5/5 and 3/7, and employed for enzymatic interesterification of PS and CnO oil, respectively (Figure 4.5). After 4h, a visible enhancement of interesterification degree by a dual lipase system could be obtained, compared with either immobilized or free lipase applied alone. Sole Lipase AK catalyzed reaction is very slow, of which the interesterification degree is less than 0.5 after 8h. Single Lipozyme TL IM yielded ID of 0.83 at 8h, in contrast all dual lipase systems produced over 0.9 reaction degree, especially, the ID of the system of 70% Lipase AK/ 30% Lipozyme TL IM amounted up to 0.95. It is known that the enzyme existed in free form is in general physical aggregates of enzyme protein (in most cases with accompanying oligosaccharides), existed as a supramolecular structure held together by noncovalent bonding (Cao et al., 2003). In terms of property, the free enzyme (herein Lipase AK) is hydrophilic. While the substrate in this study, the blend of palm stearin and CnO oil, are hydrophobic in nature and has a certain extent of viscosity at the operation temperature (60°C). Therefore, a good dispersion of free Lipase AK into oil (substrate) and a sufficient access of oil molecules to the enzyme aggregates existed in a supramolecular structure are theoretically impossible, behaving as a lower apparent activity. However, in a dual lipase reaction system, the support of the immobilized lipase possibly can also act as a carrier to adsorb co-existed free enzyme. In another word, in this dual lipase reaction system the immobilized lipase, besides acting as biocatalyst, at the same time may play the role to “immobilize” the co-existing lipase in free form. It is known that immobilization usually leads to a significant enhancement of the specific activity of enzyme because the

aggregated enzyme molecules in powder could be re-distributed or organized and located on the surface of carrier with greater specific surface area, which facilitates the efficient interaction between enzyme and substrate (Guo & Sun, 2003). Based on above assumption, one could understand the synergistic effect between free Lipase AK and immobilized TL IM.

Figure 4.6 summarized the synergistic effects of a dual enzyme system composed by Lipase AK and three different immobilized lipases with differing mixing ratios. From the presentation in Fig. 6, three observations could be generalized. Firstly, for all three immobilized lipases and in most cases, a markedly positive impact has exerted on Lipase AK. Secondly, with reaction progress the synergistic effect (or augment) gradually receded, which agrees with the observation of the dual enzyme system consisting of two immobilized lipases. The last and also the interesting one is that, with the increase of free lipase proportion in the dual enzyme system, the synergistic effects are generally becoming greater with few exceptions (AK (50%) -Novozym 435 (50%) at 4h). 70% Lipase AK with 30% immobilized lipase (for all three immobilized lipases) can achieve > 100% activity addition over the theoretical value at 2h; while the SEC values for 50% Lipase AK dual enzyme systems varied in the range of 65% - 75% at 2h (Figure 4.6). The general synergistic effects of Lipase AK with different immobilized lipases, from another angle, supported the assumption that the synergism possibly comes mainly from the “assisted immobilization” of the immobilized enzyme for co-existing the free enzyme. The receding synergism with reaction progress (Figure 4.6) could be due to the reaction closing to the equilibrium. The increase of SEC with increasing Lipase AK proportion in dual enzyme

system might be attributed to the “re-release” of the higher density of activity “hidden” within Lipase AK powder by adsorbed on co-existed immobilized lipases. Overall, free lipase mixed with immobilized lipase could generate significant synergistic effects. At certain composing ratios, this augment effect can amount up to a doubled value of the theoretical activity (Figure 4.6).

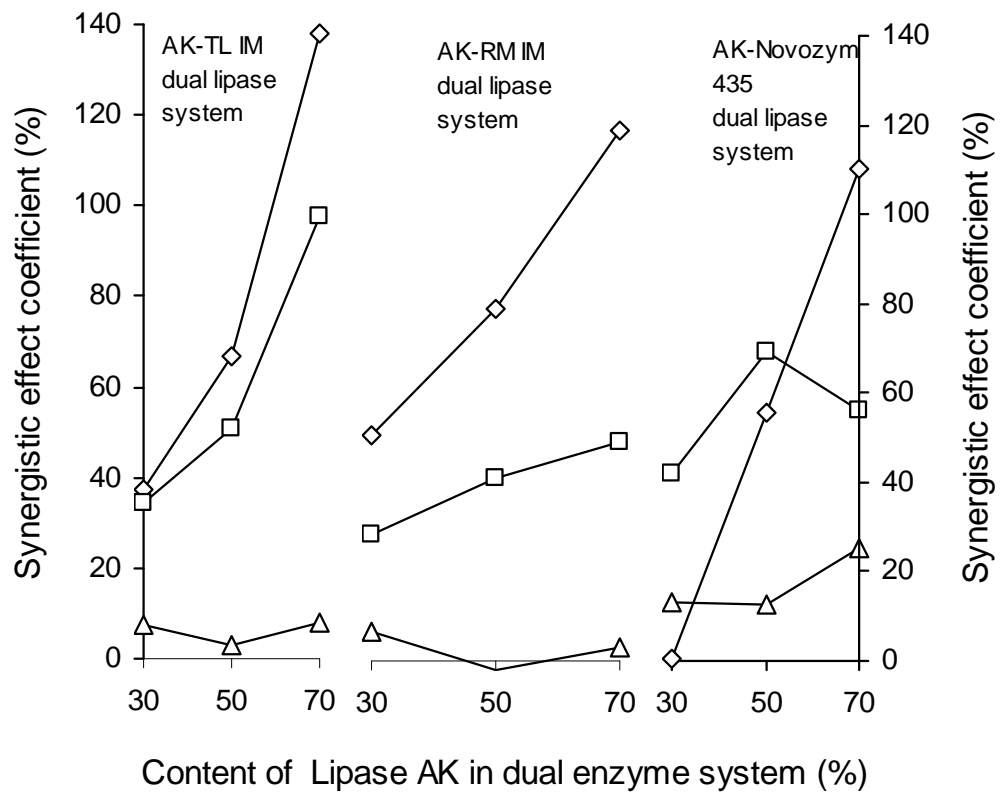


Figure 4.6 Synergistic effects of Lipase AK and different immobilized lipases mixed with different proportions. The data shown are the corresponding results at 2h (◇), 4h (□) and 8h (△).

The “assisted immobilization” effect of the immobilized lipase towards the co-existed free enzyme has been experimentally verified by the reactions catalyzed by the mixture of Lipase AK and the inactivated immobilized lipases (Figure 4.7). At 4h and thereafter, the Lipase AK mixtures with the inactivated Lipozyme TL IM and RM IM yield over 15% average conversion increase compared with sole free lipase system. The contribution of the inactivated Novozym 435 is much more significant, which achieves 60% increase at 6h and the enhancement at 8h amounts up to 84%. The differences are most likely associated with the properties of the carriers. As mentioned above, the structure of silica granule of Lipozyme TL IM has been seriously destroyed during thermal processing, which possibly accompany with the break of hydrophilic group and result in a dramatic lose of the capacity to adsorb protein. Regardless of the structural change of Lipozyme RM IM, ion-exchange resin itself is not a good absorbent for protein loading (high density of lipase in commercial Lipozyme RM IM is loaded by ion-exchange). However, as a kind of macroporous polymer, the carrier of Novozym 435 possesses a stable structure with very big specific area, which is capable of immobilizing co-existed free lipase efficiently. Therefore, this result also implies that the carrier of Novozym 435 could be a good support for lipase immobilization by simple physical adsorption.

4.3.5. Dual Lipase Systems in Continuous Operation

Figure 4.8 depicted the reaction evolutions of the interesterification performed in a PBR employing Lipozyme TL IM, Lipozyme RM IM and their mixture with ratio of 7/3 as biocatalyst, respectively. At the initial stage of the reaction, a synergistic effect of the dual lipase system has been observed (at 0.5h the experimental ID is 0.81, significantly higher

than the theoretical value, 0.62). However, after 2h the experimental ID almost overlap with the theoretical values; no apparently synergistic or antergic effect is observed. Similar results were observed for the dual lipase system of 50% Lipozyme TL IM and 50% RM IM applied to a packed bed reaction (data not shown). These results indicated that a dual lipase system in batch reaction exhibited a better synergistic effect than in continuous operation, which is probably due to an ameliorative mass transfer by convection in batch reaction but lacked in a continuous operation.

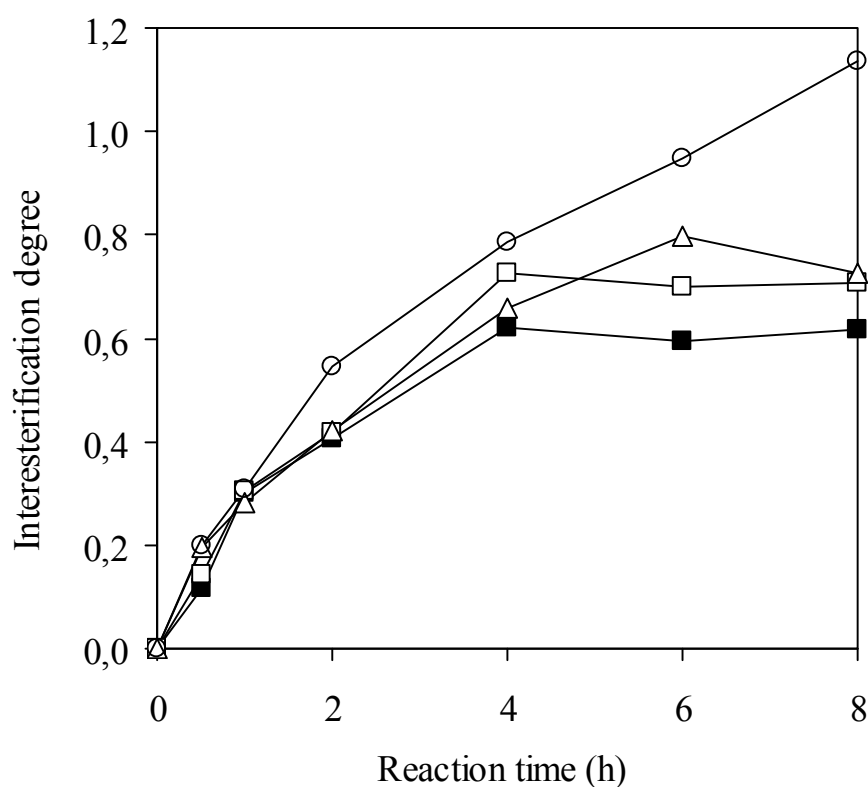


Figure 4.7 Effects of the carriers of immobilized lipases on free Lipase AK catalyzed interesterification. The biocatalyst consists of 9% (wt% of substrate) Lipase AK and the same amount of inactivated Lipozyme TL IM (□), RM IM (Δ) and Novozym 435 (○). Lipase AK without inactivated immobilized lipase (■) is used as a control.

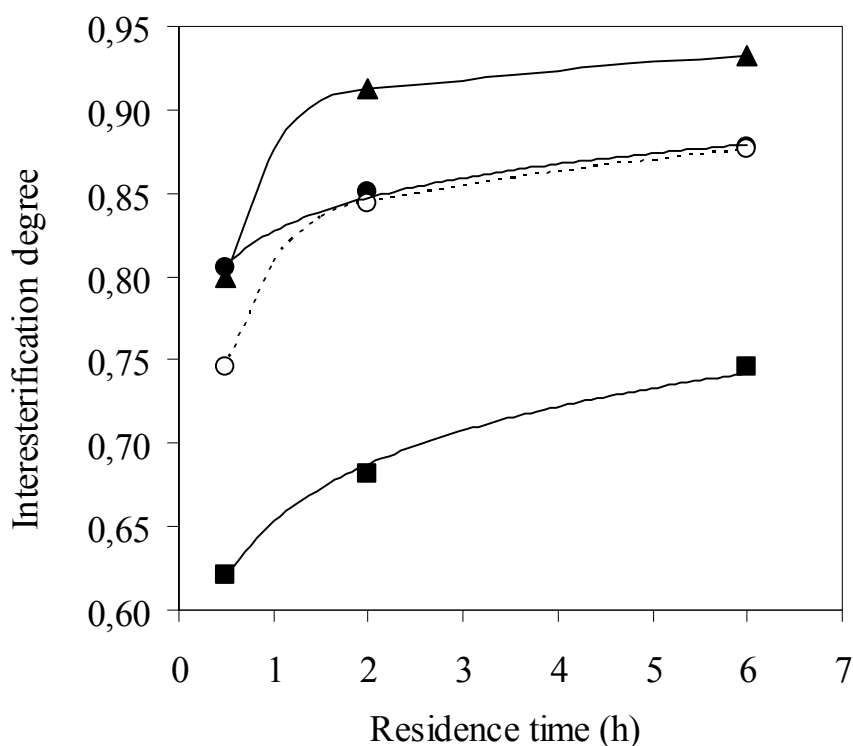


Figure 4.8 Enzymatic interesterification of palm stearin and CnO oil (7/3) in a PBR filled with sole Lipozyme TL IM (▲) or Lipozyme RM IM (■) or their mixture (5:5, w/w) (●). The theoretical interesterification degree of the dual lipase system (○) was calculated by the weighted ID sum of Lipozyme TL IM and RM IM solely acted as biocatalyst.

4.4. Conclusion

Dual enzyme system consisting of immobilized and free lipases showed a good synergistic effect possibly due to ‘assisted immobilization’ from the immobilized lipase. As the amount of free enzyme increased, the synergistic effect was also increased. In both immobilized lipases system, synergistic effects were observed in mixtures containing equal amount of lipase. Dual lipase system showed better synergistic effect in batch than continuous reaction.

Chapter 5

Improvement of Enzyme Stability with Pre-column in Continuous Packed Bed

Reactor

5.1. Introduction

Initially, enzymes were only used for producing high end products, which could be sold at a high price to compensate the high cost of the enzymes. With the availability of low cost enzymes, the applications have been widely spread into other areas such as food, detergent, structured lipids and oil and fats. A wide variety of enzymes is commercially available now; either specific or non-specific, and immobilized or non-immobilized.

There are several factors that could affect enzyme activity such as operating temperature, pH, quality and concentration of substrate. From the end users' perspective, enhancement of enzyme stability would be an advantage since this could reduce the operational cost due to a reduction in the volume of enzyme required. Another way of reducing operating cost for a batch reactor is by re-using the enzyme (Zhang et al., 2001).

Careful enzyme handling must be exercised during production, storage, transportation and application in order to have the highest possible activity and stability. The deactivation could be either reversible, where activity may be restored by the removal of the inhibitor, or irreversible, where the loss of activity is time dependent and cannot be recovered during the timescale of interest. Generally, the decay of enzyme activity is attributed to thermal effects, which causes the unfolding of the protein molecule at elevated temperature. The deactivation usually follows the first- order kinetic.

In native state, enzymes are folded into a three dimensional, compact, globular and/ or rod-like conformation of minimal free energy (Weijers and Riet, 1992). Many agents exist that affect the native state and cause denaturation. Ohta et al. (1989) reported that polymerization of lipase occurred due to the existence of hydroperoxides which led to lipase deactivation. Peroxide value greater than 5 meq/kg was found to be detrimental to enzyme lifespan (Wang and Gordon, 1991) while secondary oxidation product had a stronger effect than primary oxidation product on enzyme stability (Pirozzi, 2003). In another study, hydroperoxide compound was reported to be fully absorbed by the enzyme bed (Xu et al., 1998).

Quality of vegetable oil is normally associated with polar impurities, trace amount of metals, primary and secondary oxidation products content. Contributing factors to oxidation are amount of oxygen present, degree of unsaturation of lipids, presence of antioxidants, presence of pro-oxidants, light exposure and temperature of storage (De Man, 1999). Applications of molecular sieve in enzymatic interesterification have been reported by several authors (Ergan et al., 1988; Cerdan et al., 1998; Medina et al., 1999). The main purpose of adding the molecular sieve in a batch reactor was to remove the water formed during the enzymatic reaction. Osorio et al. (2006) reported that lipase stability decreased at a faster rate in EIE of high PUFA oil than low PUFA oil. The current study introduced a pre-column in a continuous packed bed enzymatic interesterification of an oil blend containing sunflower and fish oil which was subjected to high temperature operation to accelerate the oxidation of the blend. The purpose of introducing the pre-column into the

system is to remove any impurities from the oil blend prior to entering the enzyme bed. The deactivation rate of the enzyme bed with and without the pre-column are compared.

5.2. Material and methods

5.2.1. Materials

Refined, bleached and deodorized (RBD) sunflower oil and RBD fish oil were purchased from a local supermarket and Maritex AS, Sortland, Norway, respectively. The two oils were blended to give a 7/3 (v/v) composition ratio. Lipozyme TL IM, a silica granulated *Thermomyces lanuginosa* lipase, was donated by Novozymes A/S, Bagsvaerd, Denmark, was used for interesterification reaction in a solvent-free system. Adsorbents for the pre-column were molecular sieve, 5 Å diameter (Sigma Aldrich, Germany), activated coal (Sigma Aldrich, Germany) and bleaching earth (Sud-chemie, Germany). GLC 51B standard was purchased from Nu-Chek Prep Inc, Minnesota, USA. All chemicals and reagents for analysis were of analytical or chromatographic grades.

5.2.2. Methods

5.2.2.1. Enzymatic Interesterification

RBD sunflower and fish oil blends were subjected to EIE in a continuous PBR. The reaction was conducted in an oven which was heated to 60 °C. A stainless steel column was filled with 5.5 g Lipozyme TL IM (*Thermomyces lanuginosa*) lipase and heated to 70 °C by a circulated water bath. The column dimension was 200 mm long and 15 mm i.d. and both ends were plugged with defatted cotton. Firstly, the column was flushed with five bed volume of PS for water removal. The first sample was collected after running for another 1

h to indicate the initial activity of the enzyme. Following samples were collected through the running days from the outlet of the packed bed reactor. The samples were stored at -40 °C prior to analyses. When pre-purification was implemented before the packed bed reactor, the empty column was filled with absorbents. Approximately 6 g absorbents were packed into the column in the similar way to enzyme packing. The columns were conditioned in the same way as without absorbents through quickly pumping through 5 bed volume oil blends. Both the enzyme bed and the pre-column should be conditioned during the flushing of the oil blend. Samples were collected from the packed bed outlet in the same way as above.

5.2.2.2. Pre-column

A stainless steel column was filled with 6 g of the absorbent and fitted into the system as shown in Figure 5.1.

5.2.2.3. Analysis of TAG composition

TAG composition was determined by Hitachi-Merck HPLC Series 7000 (Hitachi-Merck, Japan). TAG components were separated by Supelcosil LC-18, 250 mm X 4.6 mm and detected by PL-ELS 2100 evaporative light scattering detector (Polymer Laboratories, Shropshire, UK). Acetone and acetonitrile acted as the mobile phase by gradient elution, began with an equal amount of the two solvents (50/50) and ended with 70% acetone and 30% acetonitrile. The mobile phase flow rate was 1.5 mL/ min.

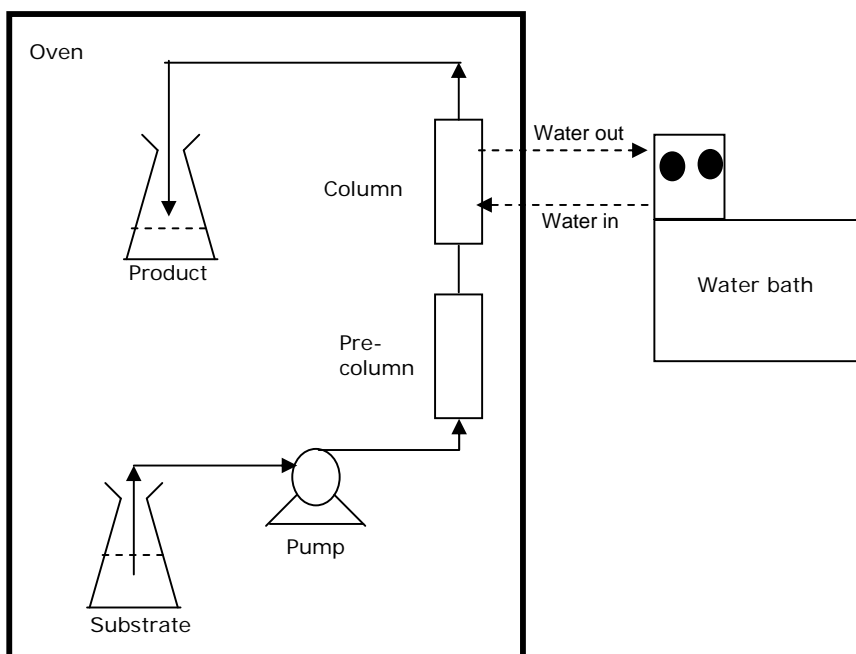


Figure 5.1. Schematic diagram of the experiment. The column was filled with lipase while the pre-column was filled with absorbent.

5.2.2.4. Interesterification degree

Interesterification degree (ID) is defined as the peak ratio between two most significantly changed peaks as described elsewhere (Rønne et al., (2005b)). The initial ID (ID_0) is then defined as the first sample collected after 1 h running. The residual activity (RA) is therefore defined as:

$$RA(\%) = \frac{ID_t - ID_B}{ID_0 - ID_B} \times 100\% \quad (\text{Eq. 1})$$

where ID_t and ID_B are the ID at running time t (h) and the ID for the oil blends without reaction, respectively.

5.2.2.5. Deactivation kinetic

The deactivation kinetics can be generally described as the first order equation as:

$$RA = RA_0 \times e^{-k_d t} \quad (\text{Eq. 2})$$

where RA_0 is initial residual activity (100% here), RA is residual activity at time t , k_d is the deactivation rate (1/h), and t is running time (h). This equation can be further described as:

$$\ln(RA) = -k_d t + \ln(RA_0) \quad (\text{Eq. 3})$$

Furthermore, the running time to reach 50% residual activity ($t_{1/2}$) can be calculated as:

$$t_{1/2} = \frac{\ln(2)}{k_d} \quad (\text{Eq. 4})$$

5.3. Results and Discussion

Generally water content has little effect on the enzyme activity as demonstrated before for Lipozyme TL IM (Zhang et al., 2001). The pre-column was not expected to affect the water content for the reaction system. For that purpose, the conditioning by flushing with the oil blend was extended to 5 bed volumes. Both molecule sieves and activated carbon were not pre-treated by drying and used as they were. Free fatty acid content was measured for the four operations for the samples after running for 1 h, with the method early described (Rønne et al., 2005b). There were no significant differences (1.4-1.6%), indicating a similar water situation in the enzyme bed system. This also implies that there was no significant drying in the pre-column with absorbents.

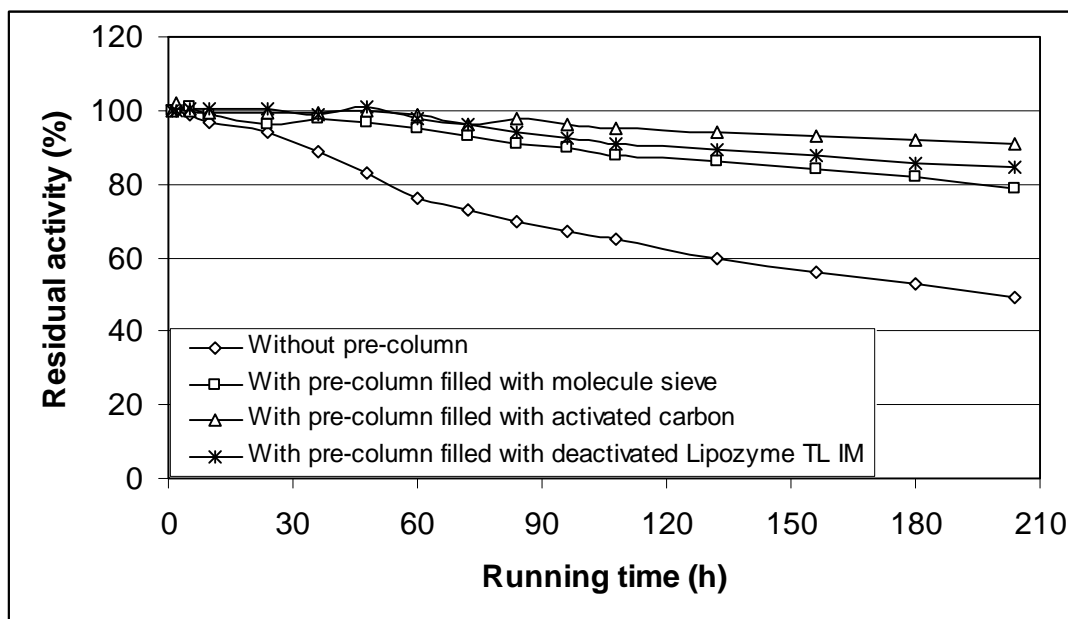


Figure 5.2. Residual activities following the continuous running of Lipozyme TL IM-catalyzed interesterification in a packed bed reactor with or without pre-column for purification. Reaction conditions: oil blend sunflower oil/fish oil (7/3 v/v), column temperature 70°C, residence time 50 min, and no additional water for the oil blend.

Table 5.1. Calculation results based on the first order deactivation kinetics

	k_d	$\ln(RA_0)$	R^2	$t_{1/2}$
Without pre-purification	0.0037	4.595	0.984	187
With pre-column filled with molecule sieves	0.0012	4.613	0.984	578
With pre-column filled with activated carbon	0.0005	4.614	0.937	1386
With pre-column filled with deactivated Lipozyme TL IM	0.0009	4.622	0.951	770

With the data in Figure 5.1, linear plots can be made following Eq. 3. The slope of the linear plot will be k_d and the intercept will be the $\ln(RA_0)$. The regression coefficients (R^2) can be also generated from the calculation to indicate the quality of the data fitting. With

k_d , $t_{1/2}$ can be therefore also calculated based on Eq. 4. Table 5.1 summarizes the calculation results. As seen from the table, the fitting was relatively satisfactory with R^2 higher than 0.937. The theoretical $\ln(RA_0)$ should be $\ln(100)=4.605$. The results in Table 5.1 are relatively close. With the results in Table 5.1, a quantitative comparison can be also made. When pre-column was filled with different absorbents, the stability can be improved by 3.1, 7.4, and 4.1 fold by filling in molecule sieves, activated carbon, and deactivated Lipozyme TL IM, respectively, in terms of k_d or $t_{1/2}$.

The capacity of the pre-column filled with the deactivated Lipozyme TL IM was also tested. The pre-column was pumped through the oil blend without further into the enzyme beds. The pre-columns after running through equivalent to 300, 600, 900, and 1200 kg oil blend per kg absorbent were subjected to the same experimental evaluation of stability with the same procedure but for one week. The results were subjected to the evaluation also with Eq. 3. k_d was found increasing in correspondent with increasing running amount of oil blend (Figure 5.2), indicating a deterioration of the pre-column absorption function. However significant change was found after 800 kg/kg absorbent. This may indicate the full saturation of the pre-column in terms of absorption.

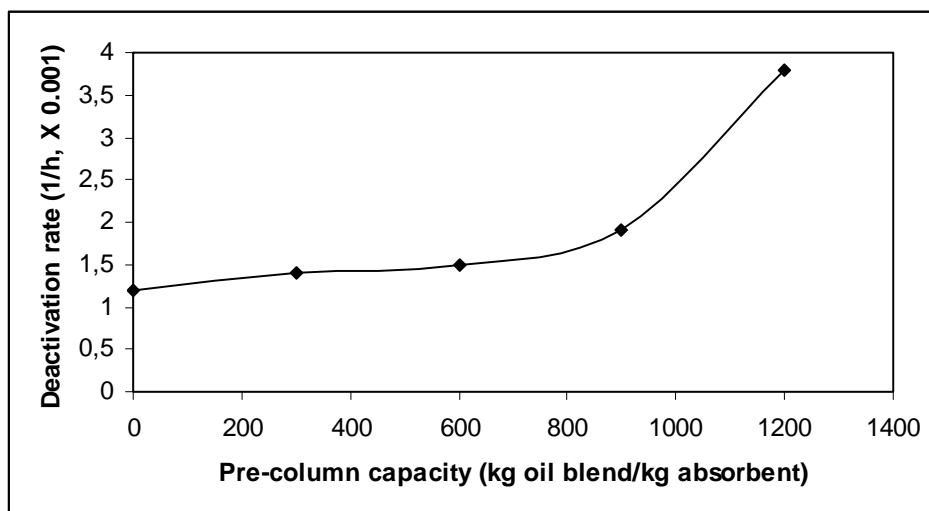


Figure 5.3. Deactivation rates (k_d) changes following the running amount of oil blend through pre-column filled with deactivated Lipozyme TL IM (pre-column capacity).

5.4. Conclusion

Lipase lifespan in a continuous PBR can be prolonged by attaching a pre-column filled with molecular sieve, activated carbon and deactivated lipase. In this set-up, the substrate would pass through the pre-column before it got in contact with the enzyme. This would ensure that any impurities in the substrate would be absorbed by the absorbent and a better quality substrate is fed to the enzyme. It is possible to prolong the lipase stability by employing a pre-column, especially when high PUFA oil involved. From economic point of view, used lipase would be a good choice to be used as absorbent in the pre-column.

Chapter 6

Overall Conclusion

In this study, production of structured lipids meant for palm-based margarine enriched with n-3 FA was carried out in a solvent-free continuous packed-bed reactor. The study covered three aspects namely margarine formulation and enzymatic interesterification, dual lipases system and stability study which are summarized below:

Margarine formulation and EIE

The addition of FO into palm products blend had an insignificant impact on the SFC of the blend. In fact, the impact of FO was almost similar to PKO. However, FO showed the greatest influence on SFC of the interesterified products due to re-arrangement of FA in the glycerol back-bone. The interesterified products contained higher solid fat than the blend, which is unique since this was against the expectation. The acyl exchange from FO to other TAGs has somehow increased the SFC. Even though the SFC could be reduced at a later stage in margarine production by adding a liquid oil, it would be interesting to know how such phenomenon could have occurred. A rheological study should be conducted to explain this unusual phenomenon.

Dual lipase system

Combinations of both immobilized and immobilized/non-immobilized lipases were proven to have a synergistic effect on the interesterification. The system with both immobilized lipases were not only shown a faster reaction, but also achieved a higher interesterification degree. Synergism was also observed in mixture of immobilized/non-

immobilized lipases. However, the current study shows that the application of dual lipase system containing both immobilized lipases seemed to be feasible only for a batch reactor. It is more economical to run a continuous reactor than batch, therefore, more research in this area is required. Also, a comparison study on the functional and physical property of single and combined lipase system should be conducted to ensure that the desired product could be achieved.

Enzyme stability

Enzyme's life-span could possibly be prolonged by applying an on-line pre-filtration. This is to ensure that some impurities in the substrate would be removed before it reaches the enzyme. From practical and economical point of view, a used lipase would be the most suitable adsorbent. This practice would prolong the enzyme's life-span and reduce the operating cost for EIE. An up-scale trial should be conducted to make sure that this practice would be feasible for the industry.

This research has initiated a production of palm-based margarine fortified with EPA/DHA via the enzymatic process targeting at health conscious consumers. In addition to the essential fatty acids, it is also free from the hazardous trans fatty acids. A dual lipase system could be applied due to the synergistic effect to offset the cost of the enzyme. However, this is limited to batch reactor only. For a continuous reactor, enzyme lifespan could be prolonged by employing a pre-filtration system where spent earth or enzyme or other adsorbents could be recycled and used as a pre-filter.

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Appendices

Appendix 1

Recycle Reactor

A preliminary work has been conducted to study the feasibility of a recycle reactor. The set-up is shown in Figure 1, where part of the interesterified product was recycled into the system. Mixing predominantly occurred instantaneously and completely at point 1 between the substrate (S) and the recycled portion (P_r). The input to the process was added to the output from the unit in the recycle loop, giving Q ($Q = S + P_r$). The variable was fed into the unit in the upward path, and the final output was P.

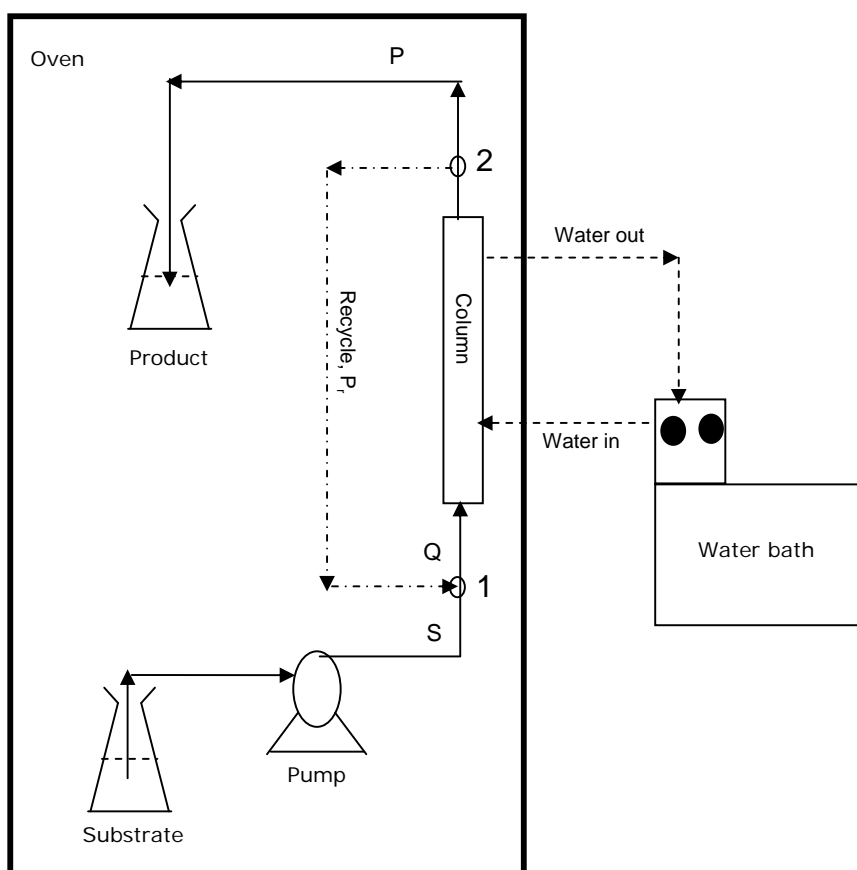


Figure 1. Experimental set-up for recycle reactor. 1: one-way valve; 2: Splitter valve

Materials and Methods

Materials

RBD PS and PKO were purchased from Golden Jomalina Food Industries Sdn Bhd, Malaysia. Lipozyme TL IM, a silica granulated *Thermomyces lanuginosa* lipase was donated by Novozyme A/S, Bagsvaerd, Denmark.

Methods

TAG analysis: As in Chapter 3.2.2.1.

Enzymatic Interesterification

Conditions are as in Chapter 3.2.2.6. The recycle ratio was adjusted to 0, 27, 45 and 60%.

Results and Discussion

Interesterification degree increased as the split ratio increased (Table 1). At higher split ratio, more interesterified product was introduced into the system, mixed with the substrate at point 1 and further interesterified by the lipase. The recycled portion still contained unreacted TAG, whereby upon interesterification of the mixture (Q) would increase the amount of reacted TAG. This preliminary result shows that it is possible to improve the interesterification performance.

Table 1. Effect of recycle on interesterification degree

Recycle %	ID
0	0.60
27	0.62
45	0.63
60	0.67

Appendix II

Triacylglycerol and Fatty Acid Content of Interesterified Products

Appendix II. Triacylglycerol Content of Interesterified Products

Margarine hardstocks were prepared by enzymatic interesterification of 13 oil blends consisting of PS (60-90%), PKO (10-40%) and FO (0-10%). EIE was carried out by a continuous packed-bed reactor filled with Lipozyme TL IM. Reaction conditions were as explained in Chapter 3.

The TAGs (determined by GC) of the interesterified products were different from the blend, as shown in Table 1. EIE caused a major increment of C44, C46 and C48 and major decrement of C50 and C52. The increment was due to acyl exchange of palmitic acid from C50 and C52 to C44, C46 and C48. Palmitic acid was the largest component in the blend (34.6-49.1 %) due to the high proportion of palm stearin in the blend, as shown in Table 2.

The increment of C44, C46 and C48 led to the higher SFC of the interesterified products than the blend at 5, 20 and 30 °C. However, the SFCs of the products were lower than the blend at temperature above 35 °C. This was also reported by Zhang et al (2004b). The current finding contradicts with those findings mentioned in Table 2.1 (page 26), where all authors except Zhang et al. (2004b) reported SFCs of interesterified products were lower than the blend. The best explanation for this phenomena is due to the replacement of sn-1 and 3 fatty acids in the TAGs with long chain saturated fatty acids from palm stearin. However, since the TAG still contained long chain PUFA, the SFC was drastically decreased at temperature above 35 °C. Also, the acyl exchange of short and medium FA from PKO contributed to the decrease in SFC.

Table 1. TAG composition of blend and interesterified products

POS/PKO/FO	C30	C32	C34	C36	C38	C40	C42	C44	C46	C48	C50	C52	C54	C56
Before EIE														
90/10/0	0.0	0.6	0.8	2.8	1.8	1.0	0.9	0.8	1.9	15.0	45.3	23.9	5.0	0.0
86,7/10/3,3	0.0	0.7	1.4	3.4	2.8	1.3	1.0	0.8	1.8	14.1	43.0	23.1	5.6	0.5
83,3/10/6,7	0.0	0.7	1.3	3.4	2.7	1.4	1.1	0.8	1.9	13.8	42.1	22.9	6.0	0.9
80/20/0	0.0	1.2	1.7	5.5	4.4	2.1	1.9	1.5	2.3	13.9	39.8	21.0	4.6	0.0
80/10/10	0.0	0.7	1.3	3.2	2.4	1.1	0.9	0.8	1.8	13.8	41.9	23.1	6.5	1.4
72,5/22,5/5	0.3	1.5	2.3	5.6	4.0	2.3	2.2	1.7	2.4	13.3	37.8	20.4	5.2	0.6
72,5/22,5/5	0.3	1.4	2.2	5.5	4.0	2.2	2.1	1.7	2.4	13.2	37.8	20.6	5.5	0.7
72,5/22,5/5	0.3	1.5	2.3	5.6	4.0	2.2	2.1	1.6	2.4	13.1	37.6	20.6	5.6	0.8
66,7/23,3/10	0.3	1.6	2.4	5.9	4.5	2.5	2.2	1.8	2.5	12.5	35.3	19.8	6.0	1.4
60/40/0	0.5	2.5	3.6	9.1	6.9	3.9	3.7	2.8	3.1	11.9	30.8	16.6	4.4	0.0
60/40/0	0.5	2.5	3.6	9.0	7.0	4.0	3.7	2.8	3.1	11.9	30.7	16.6	4.4	0.2
60/33/7	0.5	2.3	3.2	8.0	6.6	3.8	3.3	2.4	2.9	11.8	31.3	17.3	5.1	0.8
60/30/10	0.4	1.9	2.9	7.2	5.4	3.0	2.9	2.2	2.7	12.0	32.7	18.5	5.8	1.3
After EIE														
90/10/0	0.0	0.0	0.7	0.9	0.5	1.2	2.0	5.9	9.5	19.6	31.4	22.5	5.6	0.0
86,7/10/3,3	0.0	0.0	0.7	0.8	0.5	1.2	2.1	5.8	9.5	19.0	30.7	22.5	6.5	0.7
83,3/10/6,7	0.0	0.0	0.6	0.8	0.5	1.2	2.1	5.9	9.7	18.5	29.5	22.3	7.5	1.2
80/20/0	0.4	0.4	0.8	1.3	1.1	3.0	4.6	9.7	14.4	18.3	24.0	17.3	4.4	4.5
80/10/10	0.2	0.2	0.8	1.2	0.7	1.1	1.9	5.4	8.9	17.8	28.2	22.0	8.6	2.2
72,5/22,5/5	0.4	0.4	0.4	1.2	1.3	3.6	5.3	10.3	15.2	17.8	21.8	16.2	5.2	0.7
72,5/22,5/5	0.4	0.4	0.4	1.3	1.3	3.5	5.3	10.2	15.1	17.8	21.8	16.4	5.3	0.7
72,5/22,5/5	0.5	0.5	0.5	1.4	1.4	3.6	5.4	10.3	15.2	17.8	21.7	16.2	5.1	0.7
66,7/23,3/10	0.3	0.4	0.5	1.3	1.4	3.6	5.4	9.9	14.7	17.2	20.8	16.3	6.3	1.4
60/40/0	0.6	0.8	1.0	2.7	3.4	7.6	10.3	13.6	18.1	15.7	13.7	9.7	2.7	0.0
60/40/0	0.6	0.9	1.0	2.7	3.4	7.6	10.3	13.7	18.2	15.7	13.7	9.7	2.6	0.0
60/33,3/6,7	0.5	0.7	0.8	2.1	2.5	5.9	8.4	12.4	17.1	16.3	16.3	12.0	4.2	0.6
60/30/10	0.5	0.6	0.8	1.8	2.1	5.0	7.2	11.5	16.0	16.3	17.6	13.6	5.4	1.2

Table 2. Fatty acid content of interesterified products

Sample	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C20:5	C22:6
POS/PKO/FO													
90/10/0	0.5	0.4	5.6	2.8	49.1	4.2	29.7	6.6	0.1	0.1	0.1	nd	nd
86,7/10/3,3	0.5	0.4	5.7	3.0	47.8	4.1	29.1	6.3	0.2	0.4	0.5	0.2	0.4
83,3/10/6,7	0.5	0.4	6.0	3.1	45.7	4.0	28.6	6.1	0.2	0.4	1.1	0.5	0.8
80/20/0	1.0	0.8	10.8	4.3	43.9	3.9	27.4	6.9	0.1	0.4	0.1	nd	nd
80/10/10	0.6	0.5	5.6	2.8	42.3	4.2	29.8	6.1	0.3	0.7	1.4	1.3	0.8
72,5/22,5/5	1.2	0.9	12.9	5.0	41.7	3.8	27.3	5.7	nd	nd	0.7	nd	nd
72,5/22,5/5	1.0	0.8	11.7	4.8	40.7	3.8	27.0	5.8	0.2	0.4	0.8	0.4	0.6
72,5/22,5/5	1.1	0.9	12.4	4.9	40.6	3.7	26.5	5.7	0.2	0.4	0.9	0.4	0.6
66,7/23,3/10	1.0	0.8	11.5	4.9	38.2	3.6	26.4	5.7	0.2	0.4	1.6	0.8	1.2
60/40/0	2.0	1.5	21.1	7.3	34.7	3.4	24.2	5.1	nd	0.3	nd	nd	nd
60/40/0	1.9	1.5	21.1	7.2	34.6	3.4	24.1	5.3	0.1	0.3	0.1	nd	nd
60/33,3/6,7	1.6	1.3	17.9	6.5	34.6	3.4	24.3	5.1	0.2	0.3	1.0	0.5	0.8
60/30/10	1.5	1.2	16.1	6.1	34.9	3.4	24.6	5.1	0.2	0.3	1.3	0.8	1.1

Appendix III

Lipozyme TL IM Information Sheet

Product Data Sheet



Lipozyme® TL IM

Valid from 20-Sep-2005

Product Characteristics:

Enzyme Class Lipase
Declared activity 250 IUN/g

Colour can vary from batch to batch. Colour intensity is not an indication of enzyme activity.

Physical form Immobilized Granulate

Production organism *Aspergillus oryzae*

Donor organism *Thermomyces lanuginosus*

Produced by submerged fermentation of a genetically modified micro organism. The enzyme protein, which in itself is not genetically modified, is separated and purified from the production organism.

Product Specification:

	Lower Limit	Upper Limit	Unit
Interestification Units IUN	200		/g
Bulk Density	-	-	g/ml
Loss on Drying 105 C	-	8.0	%
Sieve fraction on 16 mesh	-	3.0	%
Sieve fraction through 48 mesh	-	10	%
Total Viable Count	-	50000	/g
Coliform Bacteria	-	30	/g
Enteropathogenic E.Coli		None Detected	/25g.
Salmonella		None Detected	/25g.

The product complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

Packaging: See the standard packaging list for more information.

Recommended Storage:

Best before When stored as recommended, the product is best used within 3 months from date of delivery.

Storage temperature 0-10°C (32°F-50°F)

Storage Conditions In unbroken packaging - dry and protected from the sun. The product has been formulated for optimal stability. Extended storage or adverse conditions such as higher temperature or higher humidity may lead to a higher dosage requirement.

Safety and handling precautions:

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact. Powdered enzymes are readily inhaled and should be handled only with specific precautions to prevent inhalation of dust. All equipment and handling procedures must be designed to control airborne dust. Personal respiratory protection is recommended in all cases where full dust control is not secured. All spills, however minor, should be removed immediately. Use respiratory protection. Major spills should be carefully shovelled into plastic-lined containers. Minor spills and the remains of major spills should be removed by vacuum cleaning or flushing with water (avoid splashing). Vacuum cleaners and central vacuum systems should be equipped with HEPA filters. Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes. A Material Safety Data Sheet is supplied with all products. See the Safety Manual for further information regarding how to handle the product safely.



Cost-efficient and simple edible fat production

Enzymatic interesterification is an efficient way of controlling the melting characteristics of edible oils and fats. No chemicals are used in the process and no trans fats are formed. Until recently the technology was not widely used due to the high cost of the enzyme, but now enzymatic interesterification is a cost-effective alternative to both chemical interesterification and hydrogenation.

The benefits of enzymatic interesterification are:

- **It's a cost-efficient process compared to chemical interesterification**
Neither washing nor bleaching of the interesterified fat is required, as the low temperature enzymatic process produces no side-products. (See a cost comparison on the other side.)
- **The capital investment costs are low**
The enzymatic process requires only one simple column/tank as special equipment, so the investment costs are much lower than those of both hydrogenation and chemical interesterification.
- **It's a simple and easy process**
A specific melting profile of the fat is achieved by passing the oil once through the enzyme column.
- **No trans fatty acids are produced**
Enzymatic interesterification produces no trans fatty acids, which are believed to have a negative impact on our health. In partial hydrogenation, a significant amount of the fatty acids are transformed into trans fatty acids.
- **More natural fat is produced**
The catalyst in enzymatic interesterification is a 1,3-specific lipase. The enzymes rearrange the fatty acids in the 1- and 3-positions (the 2-position is preserved). In chemical interesterification all three positions are shifted randomly.
- **It allows for a wide range of end-products**
Enzymatic interesterification enables very accurate control of the process, which allows specific melting profiles to be achieved. This means that products with new and improved melting profiles can be produced.
- **It improves industrial hygiene/safety**
Contrary to both hydrogenation and chemical interesterification, the enzymatic process requires no chemicals. The enzyme is fixed in the column throughout the production, so the only handling of the enzyme is when it is changed after the production of many hundred tonnes of fat. Enzymes are proteins. They are not hazardous to the environment nor dangerous to handle.

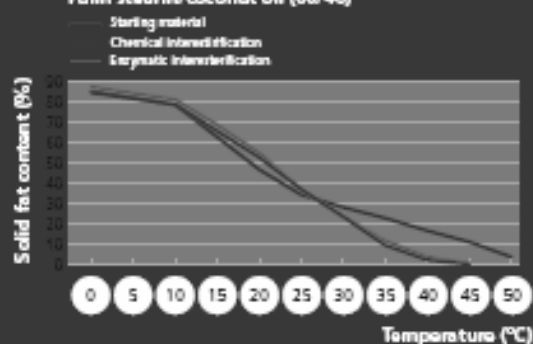

novozymes®
Unlocking the magic of nature

A calculation of process costs comparing hydrogenation, chemical interesterification and enzymatic interesterification

	Raw material price	Chemical interesterification		Enzymatic interesterification		Hydrogenation	
	Price USD/unit	Dosage unit/t oil	Cost USD/t oil	Dosage unit/t oil	Cost USD/t oil	Dosage unit/t oil	Cost USD/t oil
Intesterification							
Catalyst: NaOCH ₃	4 kg	0.5 kg	2	0 kg	0	0 kg	0
Enzyme cost USD/t oil	0/t			1	12.06	0	0
Rent of P&F					0		
Hydrogenation							
Metal	10 kg					8.6 kg	6
Hydrogen	0.3 km ³					93 m ³	16
Post-refining							
Oil loss	0.7 kg	18 kg	7	5 kg	2.1	1 kg	0.7
Bleach earth	0.5 kg	2 kg	1.5	0 kg	0	1 kg	0.5
Citric acid	1.5 kg	1 kg	1.5	0 kg	0	8.5 kg	8.75
Utilities							
Electricity	8.07 kWh	58 kWh	2.5	5 kWh	0.21	70 kWh	4.9
Steam	8.00 kg	158 kg	2	10 kg	8.2	100 kg	2
Water, air, nitrogen			0.5		8.5		8.25
Environmental							
Bleach earth	8.14 kg	2 kg	0.42	0 kg	0	2 kg	8.62
Catalyst recovery/disposal							
Maintenance							
Manpower	40000 man/yr	0.00806 man/yr	1.82	0.00026 man/yr	1.82	0.80026 man/yr	1.52
Spares parts (assumed identical)	8.85	1	0.45	1	0.45	1	8.85
Costs (USD/t of oil)			21.69		19.29		22.79

Operating costs - a comparison (data from Bellare, M., Oil Modification Processes in Edible Oil Processing, eds. Harner, W. and Hamilton, R.L., CRC Press, 2002) p. 151.)

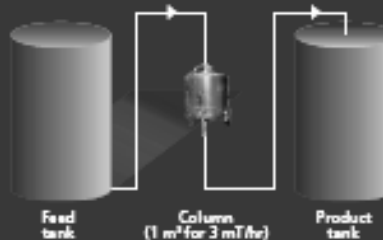
Palm stearin/coconut oil (60/40)



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Paper I

Enzymatic Interesterification of Palm Stearin and Coconut Oil by a Dual Lipase Sysytem

Enzymatic Interesterification of Palm Stearin and Coconut Oil by a Dual Lipase System

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Abstract Enzymatic interesterification of palm stearin with coconut oil was conducted by applying a dual lipase system in comparison with individual lipase-catalyzed reactions. The results indicated that a synergistic effect occurred for many lipase combinations, but largely depending on the lipase species mixed and their ratios. The combination of Lipozyme TL IM and RM IM was found to generate a positive synergistic action at all test mixing ratios. Only equivalent amount mixtures of Lipozyme TL IM with Novozym 435 or Lipozyme RM IM with Novozym 435 produced a significant synergistic effect as well as the enhanced degree of interesterification. The interesterification catalyzed by Lipozyme TL IM mixed with thermally inactivated immobilized lipase preparations indicated that the carrier property may play an important role in affecting the interaction of two mixed lipases and the subsequent reactions. A dual enzyme system, consisting of immobilized lipases and a non-immobilized one (Lipase AK), in most cases apparently endows the free lipase with a considerably enhanced activity. 70% Lipase AK mixed with 30% immobilized lipase (Lipozyme TL IM, RM IM and Novozym 435) can achieve an increase in activity greater than 100% over the theoretical value when the reaction proceeds for 2 h. The co-immobilization action of the carrier of the immobilized lipases towards the free lipase was proposed as being one of the reasons leading to the synergistic effect and this has been experimentally verified by a reaction catalyzed by a Lipase AK-inactivated preparation. No apparently synergistic effect of the combinations of Lipozyme TL IM and RM IM was observed when the dual enzyme systems applied to the continuous reaction performed in a packed bed reactor.

In brief, this work demonstrated the possibility of increasing the reaction rate or enhancing the degree of conversion by employing a dual lipase system as a biocatalyst.

Keywords Enzymatic interesterification · Dual lipase system · Synergistic effect · Interesterification degree · Lipases

Introduction

As ubiquitous enzymes, lipases (EC 3.1.1.3) constitute the most important group of biocatalysts for biotechnological applications [1, 2]. Lipases have surprising flexibility to catalyze the acylation and deacylation of a wide range of natural and unnatural substrates, which find a number of attractive applications in organic chemistry, pharmaceuticals, cosmetics and leather processing, etc. [3, 4]. However, from an industrial point of view, enzymatic modification of bulky oils and fats still represents one of the most promising technologies in terms of processing scale and practical applications [5, 6]. As one of a few commercialized approaches, enzymatic interesterification has been intensively studied [7]. The investigations cover enzyme evaluation, protocol development, fatty acid specificity, reaction optimization and packed bed reactions. These efforts have provided a lot of useful information for the better understanding of the interaction among lipase, substrates and operation parameters, and the development of practical techniques [8]. However, either in batch reaction or continuous operation, a single lipase is generally employed as the biocatalyst. Little attention has been given to dual or multiple lipase systems to reveal their synergistic or antergic effects [9].

Generally speaking, multiple enzyme systems have been assigned to successive reactions [10–12] or assistant

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actions to the main reactions by removing byproducts or enzyme pre-treatment [13, 14]. The former approach, also called combinatorial biocatalysis, can obviate some separation steps and simplify purification procedures. The latter operation could deplete the inhibiting byproducts of the enzyme for the main reaction, which would facilitate the accumulation of desired products. These investigations are not only scientifically interesting, but also result in a significantly higher enzymatic turnout than that of the enzyme employed alone [13]. This concept has been applied to crude enzyme systems (consisting of multiple enzymes), by which Barthomeuf and Pourrat [15] showed a better performance for the production of high-content fructo-oligosaccharides using crude fructosyl transferase from *Penicillium*-containing glycosidase. The utilization of combined non-specific and 1,3-specific lipases has been widely used for structured lipid production [16]. However, these consecutive syntheses were usually performed in two steps, accompanying the purification of intermediate products, in which the two types of lipases acted as biocatalysts separately. Obviously, this differs conceptually from a dual enzyme system, in which two biocatalysts acted simultaneously, with possible interactions.

The primary aim of this work was to take a close look at the possible interaction of two simultaneously occurring lipases in a dual lipase-mediated interesterification, and explore the possibility of achieving an improved reaction. As a typical system for margarine fat studies [17], palm stearin (PS) has a relatively simple fatty acid composition (oleic + palmitic > 85%) and coconut oil (CO) enriches the composition with medium-chain fatty acids (Table 1). Their blend shows a concentrated distribution of triglyceride (TG) profiles (Fig. 1a) and was therefore chosen as a model system in this study. 1,3-Specific lipases from different sources, existing in either free (Lipase from *Pseudomonas fluorescens*) or immobilized form (Lipases from *Thermomyces lanuginosa*, *Rhizomucor miehei* and *Candida antarctica* B), were employed as biocatalysts for enzymatic interesterification. The synergistic effects of dual enzymes were evaluated by the comparison of the experimental observation of individual lipase reaction systems and systems with a mixture of two enzymes. This work attempted to present new observations and a consideration of the enzymatic interesterification mediated by a dual lipase system.

Experimental Section

Materials

Palm stearin and coconut oil were purchased from Karlshamn AB, Karlshamn, Sweden. Lipozyme TL IM

Table 1 Fatty acid composition of palm stearin and coconut oil (wt%) and blend oil (PS/CO 7/3, w/w)

Fatty acid	Palm stearin	Coconut oil	Blend oil (mol%)	Blend oil (wt%) ^a
Caprylic	0.07	8.02	4.16	2.46
Capric	0.00	5.88	2.50	1.76
Lauric	0.48	47.00	17.59	14.44
Myristic	1.29	18.39	6.87	6.42
Palmitic	52.38	9.00	37.89	39.37
Stearic	4.94	2.80	3.72	4.30
Oleic	33.44	7.01	22.28	25.51
Linoleic	7.19	0.12	4.46	5.07
Linolenic	0.20	1.78	0.59	0.67

Means of triplicate determinations

^a The fatty acid compositions of the blended oils were calculated by mass balance based on the average molecular weight of palm stearin and coconut oil

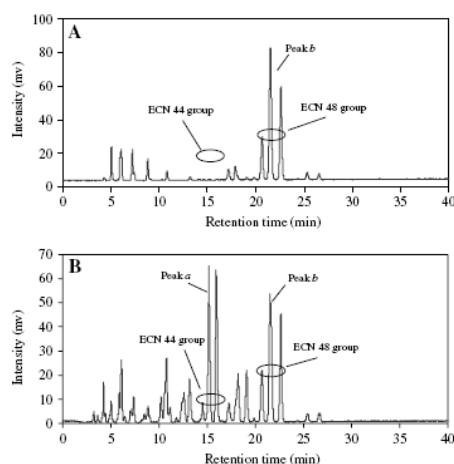


Fig. 1 HPLC chromatograms of the triglyceride profiles of palm stearin/coconut oil (7:3) blend before (a) and after 5 h enzymatic interesterification (b) catalyzed by a dual lipase system consisting of 70% (wt%) Lipozyme TL IM and 30% Lipozyme RM IM. The changes of relative contents of central peak of ECN (equivalent carbon number) 44 (*Peak a*) and 48 (*Peak b*) groups with reaction evolution, which also represent the corresponding major component of representative group, were employed as an index to monitor reaction progress

(*Thermomyces lanuginosa*), Lipozyme RM IM (*Rhizomucor miehei*), and Novozym 435 (*Candida antarctica* B) lipases were kindly provided by Novozymes A/S, Bagsvaerd, Denmark, while Lipase AK Amano 20 (*Pseudomonas*

fluorescens) was donated by Amano, Nagoya, Japan. Fatty acid methyl esters used as standards were procured from the Sigma Chemical Co. (St Louis, MO, USA). Tricaprin, trilaurin, trimyristin, tripalmitin, and tristearin used as the references for ECN determination were from Nu-Chek Prep, Inc. (Elysian, MN, USA) and with a minimum purity of 99%.

Enzymatic Interesterification

The enzymatic interesterification was conducted in a 60-mL screw-capped brown bottle and thermostated by a water bath at 60 °C. A typical reaction employed 20 g of palm stearin/coconut oil (7/3, w/w) blend, and the total amount of enzyme load was 9% (wt%) of the substrate for either sole or dual lipase systems. The reaction was initiated by the addition of lipase and agitated continuously by magnetic stirring at 300 rpm. All batch reactions were conducted under the above conditions unless stated elsewhere. Twenty-microliters of aliquots were withdrawn at desired intervals and dissolved in 1 mL of hexane assigned for HPLC analysis.

To evaluate the effects of the carriers of immobilized lipases on enzyme activity, thermal inactivation of Lipozyme TL IM, Lipozyme RM IM and Novozym 435 was carried out in a vacuum oven. The immobilized lipases were kept at 160 °C for 24 h under a vacuum. The resulting preparations were kept at room temperature for 2 h to partly recover lost moisture. The inactivated lipases were employed for interesterification of PS and CNO under the same conditions as used for active lipase to assay the residual activity. After 16 h no detectable reaction was observed for all 3 inactivated lipases, indicating the inactivated immobilized lipases can be treated as a carrier. The resulting preparations were therefore used as a replacement for an unavailable blank carrier for evaluation.

Lipozyme TL IM was used as a model to examine the effect of carrier properties on the immobilized lipase-catalyzed reaction. Equal amounts of inactivated preparation and Lipozyme TL IM were mixed for enzymatic interesterification and compared with a solely Lipozyme TL IM-catalyzed reaction. The same dosage of Lipozyme TL IM for all tests was used for comparison.

The effects of the carriers of the 3 immobilized lipases on the blended free enzyme were evaluated by the comparison of the Lipase AK-inactivated preparation blend-catalyzed interesterification with the reaction catalyzed by the same dosage of Lipase AK without inactivated immobilized lipase addition.

All reactions were performed in triplicate, and the means were used for evaluation of the results.

Determination of Fatty Acid and Triglyceride Compositions

Palm stearin and coconut oil were methylated by the boron trifluoride-methanol method [17]. Fatty acid methyl esters were analyzed on a Hewlett-Packard 5830A GC system equipped with a 25-m fused silica capillary column (25QC2/BPX/0.25 μ m film, I.D. 0.22 mm) (Scientific Glass Engineering, Melbourne, Australia) and a flame-ionization detector (FID), as well as a HP 7671A auto-sampler. The injection temperature was set at 250 °C and helium was employed as the carrier gas at a flow rate of 40 mL/min. Oven temperature programming was as follows: starting at 70 °C and held for 2 min; the temperature was increased to 210 °C at a rate of 10 °C/min and maintained at 210 °C for 5 min; and following the second increase with a rate of 40 °C/min to 250 °C and held at 250 °C for another 2 min. Fatty acid methyl ester peaks were identified by the comparison of retention times with standards.

The triglyceride compositions of materials and products were determined with a Hitachi-Merck HPLC Series 7000 (Hitachi-Merck, Japan), conjugated with a PL-ELS 2100 evaporative light scattering detector (ELSD) (Polymer Laboratories, Shropshire, UK). The reverse phase column employed was a Supelcosil LC-18 (250 mm \times 4.6 mm) (Supelcosil Inc., Bellefonte, PA). The ELSD was operated at an evaporating temperature of 70 °C and a nebulizing temperature of 50 °C with air as the nebulizing gas. Acetone and acetonitrile acted as the mobile phases by a gradient elution, beginning with an equal amount of the two solvents (50/50) and ending with 70% acetone and 30% acetonitrile. The mobile phase flow rate was 1.5 mL/min. The TG peaks were identified by the comparison of retention times with authentic triglyceride standards. Area percentages were used as weight to quantify the triglyceride composition. All measurements were conducted in triplicate.

Setup for the Evaluation of the Extent of Interesterification and the Synergistic Effect

The triglycerides of oils and fats in the HPLC chromatogram can be, in general, classified by the equivalent carbon number (ECN). The ECN depends mainly on the carbon number and the degree of unsaturation of the three bound fatty acids, of which the value is equal to the total carbon number of acyl groups with the subtraction of the number of double bonds. The triglycerides with the same ECN were able to be eluted as adjacent but separate peaks by HPLC, forming a so-called ECN group of TGs in the chromatogram (Fig. 1). For example, OOP, POP and PPP

can be eluted as a group of peaks of ECN 48 in the order as shown in Fig. 1.

As shown in Fig. 1a, the mixture of palm stearin and coconut oil exhibits characteristic TG profiles, namely, the TGs with medium chain length fatty acids are located in the retention time range of 5–10 min and the ECN 48 group of TGs of 20–23 min. According to individual HPLC analysis for PS and CO, the former are dominated by the triglycerides from the coconut oil, while the dominant ECN 48 group is represented by characteristic peaks of palm stearin [17]. Interesterification leads to the rearrangement of acyl groups within or inter-triglycerides, resulting in the changes of relative contents of TG profiles or the generation of new triglycerides [18]. The comparison of Fig. 1a and b revealed that a significant change before and after reaction is the appearance of the ECN 44 group of triglycerides (almost undetectable in the starting materials) and an evident decrease in the relative contents of the ECN 48 group of TGs. Furthermore, these two groups of triglycerides always occupy the major mass portion (>55%) during the reaction evolution. Thus, the change of relative contents of the two groups could be viewed as an index denoting the reaction evolution. Representatively, Peaks a and b are the major component belonging to respective groups. Therefore, the degree of interesterification (ID) can be simplified as

$$ID = \frac{\text{area of Peak a}}{\text{area of Peak b}} \quad (1)$$

To quantitatively evaluate the reaction performance of a dual lipase system, herein we defined the synergistic effect coefficient (SEC) as:

$$SEC(\%) = \frac{ID_{AB} - (x_A \times ID_A + x_B \times ID_B)}{x_A \times ID_A + x_B \times ID_B} \times 100\% \quad (2)$$

where ID_A and ID_B denote the individual ID of the reaction when Lipase A and B act solely as the biocatalyst. ID_{AB} is the corresponding reaction degree catalyzed by the enzyme mixture of lipase A and B with the mass fraction of x_A and x_B . For comparison, the total enzyme load of dual lipases is always the same as the amount of a single lipase in this work. Clearly, the value of SEC can be positive, if the combination effect of two lipases is ameliorative; thereby, the value can also be negative, if the effect is deteriorative. Therefore, this definition can be used to characterize a dual enzyme system qualitatively and quantitatively.

Results and Discussion

Synergistic Effects of Lipozyme TL IM and RM IM

Figure 2 presents the time courses of interesterification of PS and CO catalyzed by Lipozyme TL IM and RM IM

alone and their mixtures with different proportions. Compared with Lipozyme RM IM, Lipozyme TL IM achieved a better initial rate and a higher final degree of interesterification after 24 h, which agreed with the previous observation that Lipozyme TL IM shows a better performance for interesterification [19]. Before 4 h, the ID of dual lipases catalyzed reactions ranked between Lipozyme TL IM- and RM IM-mediated reactions, and at an identical time, the ID decrease with the increase of the RM IM proportion in the two lipase mixtures. Provided that the two lipases act separately with little interaction, this observation is reasonable because the introduction of RM IM to TL IM should result in a lower total reaction rate. Interestingly after 6 h, the dual enzyme systems, especially for the mixture of 70% TL IM and 30% RM IM, obtained a higher reaction degree (0.93 at 24 h) than the system with just Lipozyme TL IM (0.89 at 24 h).

To evaluate the synergistic effects of dual lipase systems more accurately, the theoretical ID (calculated from individual IDs of the two lipases and their proportions in the mixture) as well as the SEC of two enzyme mixtures with different ratios are depicted in Fig. 3. It is clear that for the mixtures of Lipozyme TL IM and RM IM, at any test ratio, the experimental degrees of interesterification were higher than the corresponding theoretical values. The strength of the synergistic effects was also shown to be associated with

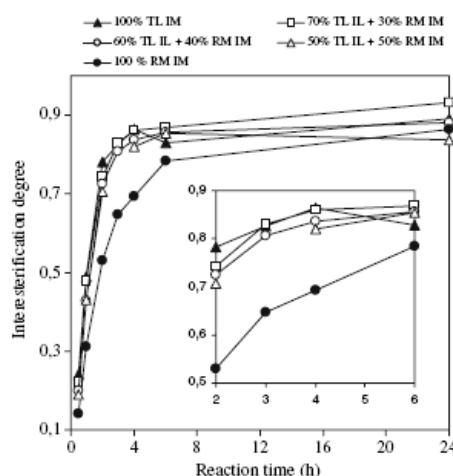
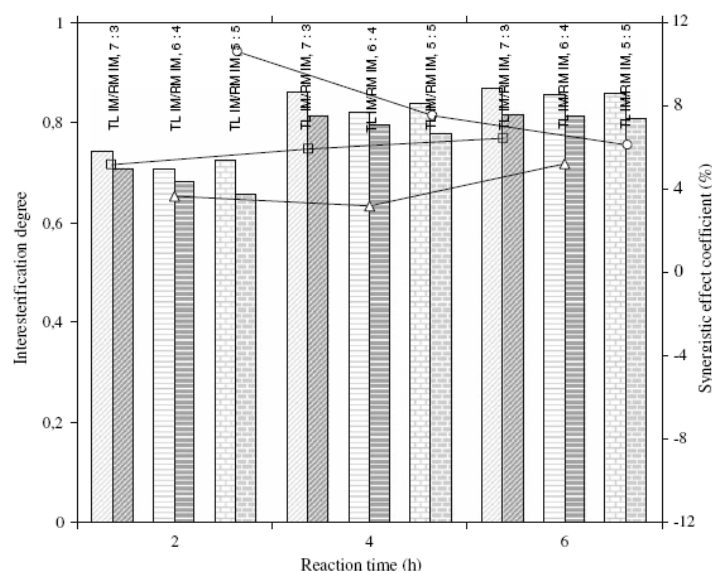


Fig. 2 Time course of enzymatic interesterification catalyzed by Lipozyme TL IM, Lipozyme RM IM, or their mixtures with different proportions. The inset is an enlarged part of the time range of 2–6 h. The batch reactions were conducted in triplicate at 60 °C with magnetic agitation. The enzyme load, either for an individual or dual lipase system was 9% (wt%) of the substrate

Fig. 3 Synergistic effects of dual enzyme systems of Lipozyme TL IM and RM IM mixed with different ratio (TL IM/RM IM). The reaction conditions were the same as in Fig. 2. The bars with a light color represent the experimental ID and those with a dark color are the corresponding theoretical values calculated by the weighted ID sum of two combined lipases when they catalyze interesterification individually. Synergistic effect coefficients (open squares, open triangles and open circles) were calculated with Eq. (2)



the ratio of two mixed lipases (Fig. 3). The SEC values of TL IM–RM IM (60:40) varied in the range of 3–5 during the time course and TL IM–RM IM (50:50) around 5.5. While TL IM–RM IM (70:30) at 2 h exhibited 10% reaction amelioration over the expected value. However, with the reaction progress this synergism comes to be closer as indicated in Fig. 3. Overall, a synergistic effect between two immobilized lipases from *Thermomyces lanuginose* and *Rhizomucor miehei* seems to be operative, even though these positive impacts are not very significant.

Interplay of Novozym 435 and Lipozyme TL IM and RM IM

To better understand the synergistic effect of a dual immobilized lipase system, we conducted similar reactions employing the dual enzyme combinations of Lipozyme TL IM–Novozym 435, and Lipozyme RM IM–Novozym 435 with different mixing ratios (Table 2). The results shown in Table 2 demonstrate that a positive interaction between two mixed lipases does not always happen. The results also indicate that the interactive effect differs with different lipase combinations and also depends on the mixing ratio. The negative SEC values of Lipozyme TL IM–Novozym 435 at the ratio of 7/3 and 3/7 at the early stage of the reaction demonstrate an antergic effect possibly occurred in this dual lipase system at the test ratios. Similar

phenomena have been observed for another dual lipase system of Lipozyme RM IM–Novozym 435 at the mixing ratio of 7/3. Interestingly, the dependency of the interactive effects on the mixing ratio of two lipases is experimentally repeatable.

It is worth noting that a significantly synergistic effect on enzymatic interesterification during the whole time course occurred, when equal amounts of Lipozyme TL IM–Novozym 435 or Lipozyme RM IM–Novozym 435 were in the mixture employed as biocatalyst. In comparison with the corresponding single enzyme system, the dual lipase system not only showed a faster reaction, but also achieved a higher degree of interesterification (Table 2).

Effects of Carriers on Lipozyme TL IM-Catalyzed Interesterification

The effects of the carriers of 3 immobilized lipases (the inactivated preparations) on Lipozyme TL IM-catalyzed interesterification are depicted in Fig. 4. Compared with sole Lipozyme TL IM-catalyzed interesterification, the contributions of co-existent inactivated Lipozyme TL IM and RM IM to the enhancement of the reaction were significant at any monitoring time. Inactivated Lipozyme TL IM generally produced over 15% ID enhancement for an identical reaction time; and the inactivated Lipozyme RM IM gave 50% enhancement of ID at 1 h and a 21% increase

Table 2 Synergistic effects of enzymatic interesterification of palm stearin and coconut oil catalyzed by dual lipase systems

Ratio of two lipases		Lipozyme TL IM: Novozym 435					Lipozyme RM IM–Novozym 435			
		10:0	7:3	5:5	3:7	0:10	10:0	7:3	5:5	3:7
2 h	^a ID	0.7819	0.3388	0.7820	0.4912	0.4675	0.5306	0.4143	0.5691	0.4760
	^b SEC (%)	–	–50.73	24.39	–12.57	–	–	–18.96	14.10	–2.12
4 h	^a ID	0.8291	0.6557	0.8763	0.7263	0.6967	0.7847	0.6979	0.8398	0.8797
	^b SEC (%)	–	–26.10	14.85	–1.40	–	–	–3.06	18.29	24.50
8 h	^a ID	0.8311	0.8826	0.9318	0.8567	0.9023	0.8012	0.8788	0.9277	0.9543
	^b SEC (%)	–	3.54	7.51	–2.75	–	–	5.64	8.92	9.46

^a Means of triple batch experiments. 9% of substrate was used as the dosage of biocatalyst for either single or dual lipase systems and all other conditions were identical

^b The synergistic effect coefficient (SEC) was calculated by Eq. (2)

at 4 h, respectively. However, the inactivated Novozym 435 showed a different behavior, i.e. a positive contribution before 2 h and a negative effect at 4 h and 6 h as well as a ID close to that with just Lipozyme TL IM at 8 h.

It should be pointed out that, after inactivation processing at higher temperatures, the structure of Lipozyme TL IM could have changed, as the color changed to brown. The resulting preparation showed good mixing with Lipozyme TL IM, behaving as a hydrophobic carrier. In contrast, the structure of the carrier of Novozym 435 seems to change little (No color change observed). Aggregation was observed when mixed with Lipozyme TL IM, showing a more hydrophilic property. No apparent change for inactivated Lipozyme RM IM could be seen (due to the original brown color) and a good mixing with Lipozyme TL IM was also observed. These observations are not only able explain the results in Fig. 4 but also support our assumption that the dispersion state of immobilized lipases may affect dual lipase-catalyzed reactions. A hydrophobic carrier could be well dispersed in the oil blend and help the dispersion of the co-existent lipase, while a hydrophilic carrier is not good for dispersion in a hydrophobic reaction mixture and also induces the aggregation of co-existent immobilized lipase. This might also help us understand why the synergistic effect was always observed for the Lipozyme TL IM–RM IM dual system (Fig. 3) but not always for the Lipozyme TL IM–Novozym 435 system (Table 2), if one considers the hydrophobic property of the RM IM carrier differing from the carrier of Novozym 435.

Dual Enzyme System of Free and Immobilized Lipases

To examine the effects of different occurring forms of enzymes in a combining system, Lipase AK Amano 20 (powder) was mixed with Lipozyme TL IM at the ratio of 7/3, 5/5 and 3/7, and employed for enzymatic interesterification of palm stearin and coconut oil, respectively

(Fig. 5). After 4 h, a visible enhancement of the degree of interesterification by a dual lipase system was obtained and compared with either immobilized or free lipase applied alone. The reaction catalyzed by Lipase AK alone is very slow and the degree of interesterification is less than 0.5 after 8 h. Lipozyme TL IM alone yielded an ID of 0.83 after 8 h. In contrast, all dual lipase systems produced a degree of reaction of over 0.9, the ID of the system with 70% Lipase AK/30% Lipozyme TL IM amounted to as much as 0.95. It is known that the enzyme in its free form is, in general, physical aggregates of enzyme protein (in most cases with accompanying oligosaccharides), existing

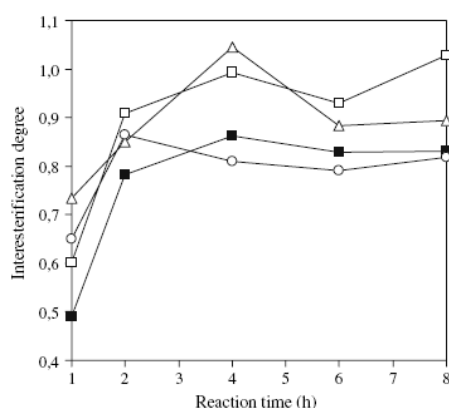


Fig. 4 Effects of the carriers of immobilized lipases on Lipozyme TL IM catalyzed interesterification. The interesterification was conducted with 10 g substrate (7/3, PS/CNO) at 60 °C and with agitation at 300 rpm. The biocatalyst consisted of 9% (wt% of substrate) Lipozyme TL IM and the same amount of inactivated Lipozyme TL IM (open squares), RM IM (open triangles) and Novozym 435 (open circles). The Lipozyme TL IM without immobilized lipase (filled squares) was used as a control

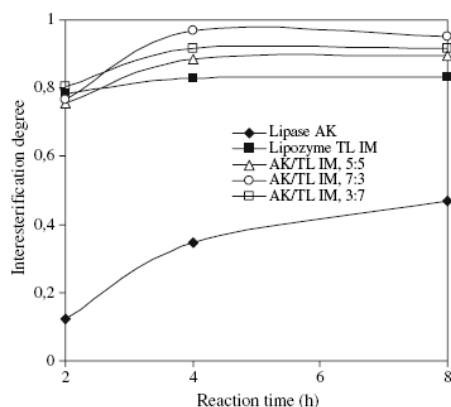


Fig. 5 Synergistic effects of free (Lipase AK) and immobilized (Lipozyme TL IM) lipases. The total amount of free or immobilized enzyme as well as their mixtures are 9% (wt%) of the substrate

as a supramolecular structure held together by noncovalent bonding [20]. In terms of its properties, the free enzyme (here Lipase AK) is hydrophilic. While the substrate in this study, the blend of palm stearin and coconut oil, is hydrophobic in nature and has a certain amount of viscosity at the operating temperature (60 °C). Therefore, a good dispersion of free Lipase AK in the oil (substrate) and a sufficient access of the oil molecules to the enzyme aggregates existing in a supramolecular structure are theoretically impossible, and thus it has a low apparent activity. However, in a dual lipase reaction system, the support of the immobilized lipase can possibly also act as a carrier to adsorb co-existing free enzyme. In other words, in this dual lipase reaction system, the immobilized lipase, besides acting as a biocatalyst, at the same time may play the role of “immobilizing” the co-existing lipase in free form. It is known that immobilization usually leads to a significant enhancement of the specific activity of an enzyme because the aggregated enzyme molecules in powder form could be re-distributed or organized and located on the surface of the carrier thus having a greater specific surface area, which facilitates the efficient interaction between enzyme and substrate [21]. Based on the above assumption, one can understand the synergistic effect between free Lipase AK and immobilized TL IM.

Figure 6 summarizes the synergistic effects of a dual enzyme system composed of Lipase AK and three different immobilized lipases with differing mixing ratios. From the presentation in Fig. 6, three observations can be generalized. Firstly, for all three immobilized lipases and in most cases, a markedly positive impact has been exerted on

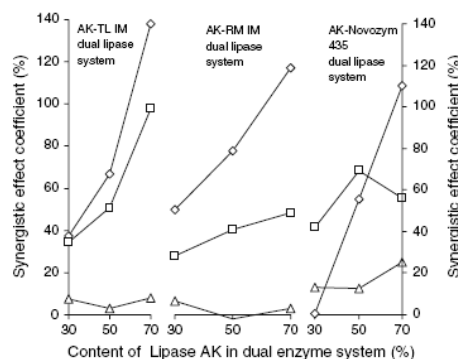


Fig. 6 Synergistic effects of Lipase AK and different immobilized lipases mixed with different proportions. The data shown are the corresponding results at 2 h (open diamonds), 4 h (open squares) and 8 h (open triangles)

Lipase AK. Secondly, with reaction progress, the synergistic effect gradually recedes, which agrees with the observation that the dual enzyme system consists of two immobilized lipases. The last and also the most interesting observation is that, with the increase of the free lipase proportion in the dual enzyme system, the synergistic effects generally become greater with few exceptions [AK (50%) -Novozym 435 (50%) at 4 h]. Seventy percent of Lipase AK with 30% immobilized lipase (for all three immobilized lipases) can achieve >100% activity enhancement over the theoretical value at 2 h; while the SEC values for 50% Lipase AK dual enzyme systems varied in the range of 65–75% at 2 h (Fig. 6). The general synergistic effects of Lipase AK with different immobilized lipases, from another angle, supported the assumption that the synergism possibly comes mainly from the “assisted immobilization” of the immobilized enzyme for the co-existing free enzyme.

The “assisted immobilization” effect of the immobilized lipase towards the co-existing free enzyme has been experimentally verified by the reactions catalyzed by the mixture of Lipase AK and the inactivated immobilized lipases (Fig. 7). At 4 h and thereafter, the Lipase AK mixtures with the inactivated Lipozyme TL IM and RM IM yielded an over 15% average conversion increase compared with sole free lipase system. The contribution of the inactivated Novozym 435 was much more significant, achieving a 60% increase at 6 h and the enhancement at 8 h came to as much as 84%. The differences are most likely associated with the properties of the carriers. As mentioned above, the structure of the silica granules of Lipozyme TL IM is destroyed during thermal processing, possibly accompanied by the break of hydrophilic group

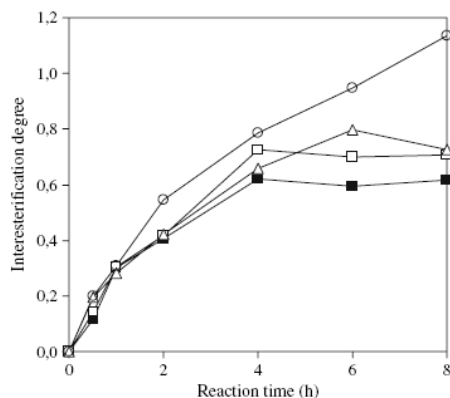


Fig. 7 Effects of the carriers of immobilized lipases on free Lipase AK catalyzed interesterification. The interesterification is conducted with 10 g substrate (7/3, PS/CNO) at 60 °C and with agitation at 300 rpm. The biocatalyst consists of 9% (wt% of substrate) Lipase AK and the same amount of inactivated Lipzyme TL IM (*open squares*), RM IM (*open triangles*) and Novozym 435 (*open circles*). Lipase AK without inactivated immobilized lipase (*filled square*) is used as a control

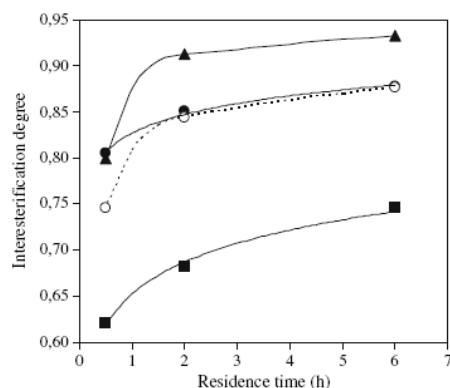


Fig. 8 Enzymatic interesterification of palm stearin and coconut oil (7/3) in a packed bed reactor filled with just Lipzyme TL IM (*filled triangles*) or just Lipzyme RM IM (*filled squares*) or a mixture of the two (7/3, *w/w*) (*filled circles*). The theoretical interesterification degree of the dual lipase system (*open circles*) was calculated by the weighted ID sum of Lipzyme TL IM and RM IM acting solely as the biocatalyst

and resulting in a dramatic loss of the capacity to adsorb protein. Regardless of the structural change of Lipzyme RM IM, the ion-exchange resin itself is not a good adsorbent for protein loading (the high density of lipase in

commercial Lipzyme RM IM is loaded by ion-exchange). However, as a kind of macroporous polymer, the carrier of Novozym 435 possesses a stable structure with very large specific area and is capable of immobilizing co-existed free lipase efficiently. Therefore, this result also implies that the carrier of Novozym 435 could be a good support for lipase immobilization by simple physical adsorption.

Dual Lipase Systems in Continuous Operation

Figure 8 depicts the reaction evolutions of the interesterification performed in a packed bed reactor employing Lipzyme TL IM, Lipzyme RM IM and their mixture with a ratio of 7/3 as biocatalyst, respectively. At the initial stage of the reaction, a synergistic effect of the dual lipase system was observed (at 0.5 h the experimental ID is 0.81, significantly higher than the theoretical value, 0.62). However, after 2 h the experimental ID almost overlapped the theoretical values; no apparently synergistic or antergic effect was observed. Similar results were observed for the dual lipase system of 50% Lipzyme TL IM and 50% RM IM applied in a packed bed reaction (data not shown). These results indicated that a dual lipase system in a batch reaction exhibited a better synergistic effect than in continuous operation, which is probably due to an ameliorative mass transfer by convection in the batch reaction but which is lacking in a continuous operation.

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Paper II
Online Pre-purification for the Continuous Enzymatic Interesterification of Bulk Fats
Containing Omega-3 Oil

Online Pre-purification for the Continuous Enzymatic Interesterification of Bulk Fats Containing Omega-3 Oil

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Dear Sir

There has been dramatic progress in the last few years in the technology development and scientific advancement in the lipase-catalyzed modification of bulk fats such as those used for margarine production [1–5]. It has been one of the major breakthroughs that enzyme technology can be used for processing of common foods economically, and imparts other potential advantages such as less pollution, simplified processes, improved product quality, etc. The technology has been increasingly utilized in industry [6].

One of the poorly understood issues of the technology is the reduction of catalyst activity following the reuse of enzymes in industrial operations. This is different from chemical interesterification using a chemical catalyst, where no such phenomenon occurs in industrial operations. We now know that enzyme function can be affected not only by the inherent characteristics of the lipase such as thermostability and optimal water activity but also by many external factors such as material/oil quality and process parameters.

Oil quality in reality can vary with the source, and is a function of processing operations. We know a few factors are inherent to oil quality such as metal content, peroxide content, polymer content, gum content, etc. In the native state, enzymes are folded into a three dimensional, compact, globular and/or rod-like conformation of minimal free energy. Many of the above-mentioned agents can affect the native state and cause denaturation. Ohta et al. [7] reported that polymerization of lipase occurred due to the presence

of hydroperoxides which led to lipase deactivation. Peroxide values greater than 5 mequiv/kg were found to be detrimental to enzyme activity retention [8], while, in another study, secondary oxidation products had a stronger effect than primary oxidation products on enzyme stability [9]. Furthermore, hydroperoxide compounds as well as other impurities such as polymers, gums, etc. were reported to be fully or partially absorbed by the enzyme bed [10]. All these studies suggest that better quality in terms of low oxidation and other detrimental components is needed for improved stability of the lipases during reuse. For all of these reasons, a refined, bleached, and deodorized (RBD) oil has been recommended for practical operations of the enzymatic interesterification processes [11].

To improve nutritional quality, the need to increase the omega-3 polyunsaturated fatty acyl content of bulk oil for products such as margarine has gained attention. This is particularly relevant to the use of enzyme processing for such modifications where milder conditions of the enzyme process can be really beneficial for the protection of omega-3 oils against oxidation. Omega-3 oils are very sensitive to oxidation and can occur during the process operation. Therefore, even using an RBD oil may be insufficient to maintain the enzyme stability, as demonstrated in a recent study where the increase of omega-3 oil in the substrate reduced the stability of the lipase used [12]. Therefore, an additional solution is imperative. One solution is an on-line monitoring process to closely follow the change of reaction performance [13, 14]. In addition, an on-line pre-purification step to remove impurities from the substrates prior to their introduction to the bioreactor would be useful to increase the stability of lipase since oxidation can occur in purified oils during storage.

Therefore, in this study, an online pre-purification step was implemented through a pre-column upstream of the

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packed bed reactor for the enzymatic interesterification. The oil blends employed as substrate contained sunflower and fish oils in order to accelerate the possible oxidation and make the performance of pre-purification significant. Two common absorbents, molecular sieves and activated carbon, were used for this purpose. In addition a bed containing spent immobilized lipase for pre-cleaning was also employed for practical process considerations. The deactivation rates of the enzyme bed with and without the pre-column were compared.

Refined, bleached and deodorized (RBD) sunflower oil was purchased from a local supermarket (peroxide value 2.6 mequiv/kg, water content 0.06 wt%, free fatty acid content 0.03%, and fatty acyl composition C16:0 6.0, C16:1 0.3, C18:0 4.6, C18:1n-9 17.8, C18:2n-6 69.2, others 2.1). RBD fish oil was obtained from Maritex AS, Sortland, Norway (peroxide value 4.1 mequiv/kg, water content 0.06 wt%, free fatty acid content 0.12%, and fatty acid composition C14:0 8.0, C15:0 0.6, C16:0 14.0, C16:1 4.6, C17:0 0.7, C17:1 0.7, C18:0 2.0, C18:1n-9 13.0, C18:1n-7 1.9, C18:2n-6 2.7, C18:3n-3 2.3, C18:4n-3 8.7, C20:1 4.2, C20:5n-3 10.4, C22:1n-11 7.6, C22:5n-3 1.3, C22:6n-3 16.2, others 1.1). The two oils were blended to give a 7/3 (v/v) composition ratio. Lipozyme TL IM, a silica granulated *Thermomyces lanuginosa* lipase, was donated by Novozymes A/S, Bagsvaerd, Denmark (water content 5.8 wt%). Adsorbents for the pre-column were molecular sieves (5 Å diameter), activated carbon (50 mesh and 900–1,100 m²/g surface area, Sigma, St Louis, MO), and deactivated Lipozyme TL IM. The deactivation was conducted at 120 °C for 24 h. The deactivated lipase was checked through interesterification with the oil blend and no significant change of triacylglycerol profile was observed. Triacylglycerol standard was purchased from Nu-Chek Prep Inc., Minnesota, USA. All chemicals and reagents for analysis were of analytical or chromatographic grades.

The oil blend was subjected to enzymatic interesterification in a continuous packed bed reactor, a jacketed stainless steel column filled with 5.5 g Lipozyme TL IM (*Thermomyces lanuginosa*) lipase and heated to 70 °C by a circulatory water bath. The column dimension was 200 mm long and 15 mm in internal diameter and both ends were plugged with defatted cotton. An empty column of the same dimensions was connected before the enzyme bed. Both columns were heated to 70 °C by the same water bath. The columns were first flushed with five bed volumes of the oil blend for enzyme conditioning by a metering pump (Fluid Metering Inc., New York, NY) with an upward flow of approximately 1 mL/min [12]. The residence time for the enzyme bed was then adjusted to 50 min through the change of substrate feeding rate. The first sample was collected after operating for 1 h to indicate the

initial activity of the enzyme, to insure that the enzyme bed was properly conditioned initially [12]. Additional samples were collected daily from the outlet of the packed bed reactor during the operation of the bioreactor system. The samples were stored at –40 °C prior to analysis. When pre-purification was implemented before the packed bed reactor, the empty column was filled with absorbents. Approximately 6 g absorbents were packed into the column in the similar way to enzyme packing. The columns were conditioned in the same way as without absorbents by quickly pumping five bed volumes of the oil blends. Both the enzyme bed and the pre-column were simultaneously conditioned during the flushing of the oil blend. Samples were collected from the packed bed outlet in the same way as described above.

The triacylglycerol profile of samples was determined using a Hitachi–Merck HPLC Series 7000 (Hitachi–Merck, Japan) system with a Supelcosil LC-18, 250 mm × 4.6 mm column and PL-ELS 2100 evaporative light scattering detector (Polymer Laboratories, Shropshire, UK). The mobile phase consisted of acetone and acetonitrile at 1.5 mL/min, with the ratio of the former to the latter varying linearly with the gradient rate of 0.5 volume change per min from 50/50 to 70/30 v/v during a 40 min analysis time. The degree of interesterification (ID) is defined as the peak ratio between two most significantly changed peaks as described elsewhere [4]. The initial ID (ID₀) is then defined as the ID value for the sample collected at 1 h. The residual activity (RA) is therefore defined as:

$$RA(\%) = \frac{ID_t - ID_B}{ID_0 - ID_B} \times 100\% \quad (1)$$

where ID_t and ID_B are the ID at operational time *t* (h) and for the oil blends prior to their contact with lipase, respectively.

Both oils used in the blend were RBD with reasonably low peroxide values (2.6–4.1 mequiv/kg). From a commercial point of view, the oils would be regarded as high quality. However, sunflower oil contains about 70% linoleic acid and fish oil about 30% omega-3 polyunsaturated fatty acids. The latter is highly sensitive to oxidation. As expected, the residual activity decreased steadily during 200 h of operational time (Fig. 1) and reached 50% at the end of the operation. The phenomenon was confirmed by repeated trials. When selected absorbents were employed in the pre-column, the stability was greatly improved (Fig. 1). There were slight differences between the absorbents used. However, the improvements were generally significant.

The absorbents selected were not arrived at by extensive screening but with the simple aim to absorb polar compounds resulting from oxidation, residual metal elements,

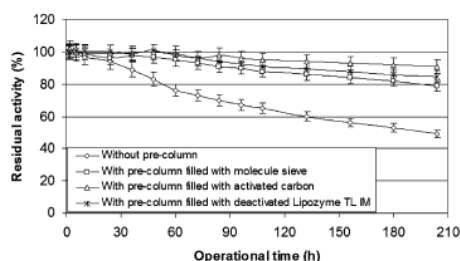


Fig. 1 Residual activities following the continuous operation of Lipozyme TL IM-catalyzed interesterification in a packed bed reactor with or without a pre-column. Reaction conditions: oil blend sunflower oil/fish oil (7/3 v/v), column temperature 70 °C, residence time 50 min, and no additional water for the oil blend. Other conditions are given in the text

gums, etc. after RBD processes. From a practical and economical point of view, the use of a spent enzyme bed as a pre-purification stage will be feasible because it serves as a waste product. This will be viable for practical processes as well. In this case, we conducted the experiments using Lipozyme TL IM artificially deactivated by heating. The absorption behaviour of the spent lipase could differ from that of the active enzyme due to differences in water content or protein structural state. After conditioning with the oil blend, we believe that the absorption performance should not be significantly different. Certainly this should be investigated further.

In the present study, the possible effect of water content on the stability was also discussed. Generally, water content has little effect on the enzyme activity for Lipozyme TL IM as demonstrated previously [2]. To reduce the effect of the water content, the conditioning by flushing with the oil blend was extended to five bed volumes from the three normally used. Both molecular sieves and activated carbon were not pre-treated by drying and used as they were. We measured the free fatty acid content for the four experimental runs for the samples after operating for 1 h, with the method previously described in [4]. There were no significant differences (1.4–1.6%), indicating a similar water activity in the enzyme bed system in the presence versus

absence of the pre-columns. This also implies that there was no significant drying of the pre-column's absorbents.

Deactivation can be generally described by a first-order kinetic equation:

$$RA = RA_0 \times e^{-k_d t} \quad (2)$$

where RA_0 is initial residual activity (100% here), RA residual activity at time t , k_d the deactivation rate (h^{-1}), and t is running time (h). Upon rearrangement, this equation can be described as:

$$\ln(RA) = -k_d t + \ln(RA_0) \quad (3)$$

Furthermore, the operational time to reach 50% residual activity ($t_{1/2}$) can be calculated as:

$$t_{1/2} = \frac{\ln(2)}{k_d} \quad (4)$$

With the data in Fig. 1, linear plots can be made that correspond to Eq. 3 that yield k_d (and hence $t_{1/2}$ via Eq. 4) and $\ln(RA_0)$ from the slope and intercept, respectively. Table 1 summarizes the calculation results. As seen from the table, the fitting was relatively satisfactory with R^2 values higher than 0.937. The theoretical $\ln(RA_0)$ value, $\ln(100) = 4.605$, corresponds well with the calculated values. When pre-column was used, the stability was improved by 3.1, 7.4, and 4.1-fold using molecular sieves, activated carbon, and deactivated Lipozyme TL IM, respectively, in terms of k_d or $t_{1/2}$.

The capacity of the pre-column filled with the deactivated Lipozyme TL IM was also tested. The oil blend was pumped through the pre-column without passing through the enzyme beds. The pre-columns after contacting 300, 600, 900, and 1,200 kg oil blend per kg absorbent were subjected to the same experimental evaluation of stability with the same procedure described above but for 1 week. k_d was found to increase proportionally to the amount of oil fed to the column per unit mass of absorbent (Fig. 2), indicating a deterioration of the pre-column performance. However, a major decrease was found employing 800 kg/kg absorbent. This may indicate the full saturation of the pre-column's absorption sites.

Table 1 Calculation results based on the first order deactivation kinetics

	k_d (h^{-1}) ^a	$\ln(RA_0)$ ^a	R^2 ^a	$t_{1/2}$ (h) ^b
Without pre-purification	0.0037 ± 0.0002	4.595	0.984	187 ± 11
With pre-column filled with molecular sieves	0.0012 ± 0.0001	4.613	0.984	578 ± 48
With pre-column filled with activated carbon	0.0005 ± 0.00003	4.614	0.937	1386 ± 83
With pre-column filled with deactivated Lipozyme TL IM	0.0009 ± 0.00004	4.622	0.951	770 ± 34

^a Calculation based on Eq. 3. R^2 is the linear regression coefficient representing goodness of linearities for Eq. 3 with experimental results

^b Calculated based on Eq. 4

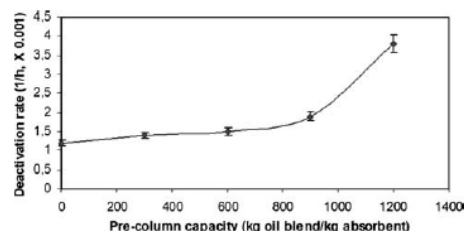


Fig. 2 Deactivation rates (k_d) versus amount of oil blend fed to pre-column filled with deactivated Lipozyme TL IM (pre-column capacity). Operational conditions given in Fig. 1

In general, this study has demonstrated the practicality of employing an online pre-purification stage for the stabilization of the enzyme bed. In particular, the use of a spent enzyme bed could be economical and practically feasible. The study used a highly unsaturated oil blend in order to make the study significant. For less unsaturated oil blends, the performance needs to be demonstrated further. The use of actual rather than simulated spent enzyme for this purpose needs to be investigated.

Acknowledgments The financial support from the Malaysian Palm Oil Board (MPOB) and the Center for Advanced Food Studies (LMC) is gratefully acknowledged.

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Enzymatic Interesterification of vegetable oil/fish oil blend for margarine production



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Introduction

Margarine can be produced either by blending, hydrogenation or interesterification (enzymatic or chemical). Previously, partial hydrogenation was a common method for margarine production. The process has been ceased from operation in margarine plant due to formation of trans fatty acid which is known to be carcinogenic. Chemical interesterification is also a popular method but disposal of nickel catalyst poses hazard to the environment. Enzymatic interesterification has gained a lot of attention lately due to the regiospecificity of lipases which enable the production of structured lipids (SL). SL can be designed to improve physical characteristics of fats, such as melting behavior or plasticity. Furthermore, it is also possible to improve the nutritional value of fats by restructuring the triacylglycerol (TAG) in such a way that the long chain n-3 poly-unsaturated fatty acids (PUFA) are located at the sn-2 position while the medium chain fatty acids (MCFA) are located at the outer positions. The former would become a source of essential fatty acid (EFA) after being absorbed through the intestinal wall whilst the latter would be consumed as a quick source of energy. Another advantage of enzymatic modification is that it is a green technology since lipase is biodegradable.

Objectives

- To study the effect of fish oil on crystallization behaviour, SFC, melting point and oxidative stability of the blend and interesterified product.
- To restructure the TAG by locating EPA/DHA at sn-2 position while MCFA at the end positions.

Experimental set-up

Material

Oil blend: Palm stearin/Palm kernel oil/Fish oil
Ratio (wt%): 60 - 90 / 10 - 40 / 0 - 15
Enzyme: Lipozyme TL IM lipase

Procedure

As shown in Figure 1.

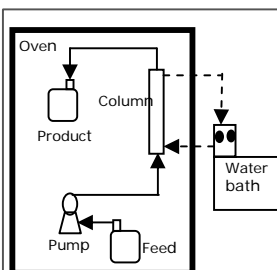


Figure 1. Experimental

Reaction condition

Oven temp: 57 °C
Water bath temp: 70 °C
Residence time: 2 hours
Enzyme weight: 12.8 g
Column size: 15 X 20 mm

TAG analysis

Instrument: HP 5890 GC
Injection: On-column
Oven: 90-360 °C
Ramp: 20 °C/min
Detector: FID
Det temp: 380 °C

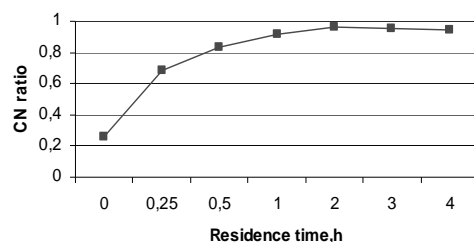


Figure 2. Time course for the reaction

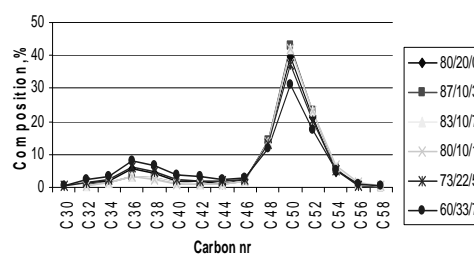


Figure 3. TAG profile of the blend before esterification.
Oil composition (%) of the blend is shown as PS/PKO/FO

Results

- The maximum interesterification degree was achieved in residence time of 2 hours (Figure 2), which was applied for all reactions.
- As the ratio of FO increased, C52 and other longer chain TAGs content also increased (Figure 3). This would increase the degree of unsaturation of the blend, thus influence the

SFC, melting point and oxidative stability.

Future work

- To determine the physico-chemical properties of the blend and product
- To study the influence of FO on SFC, melting point and crystallization property

Acknowledgement
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Malaysian Palm Oil
Board

Formulation of functional palm-based margarine by enzymatic interesterification

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Eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids are categorized as omega-3 fatty acids, and also known as essential fatty acids (EFA) due to their beneficial effect to human health. Marine oil is a major source for such fatty acids (FA), which normally located at sn-2 position in a triacylglycerol (TAG) backbone. A lot of studies have been conducted on those fatty acids, however, the effect of adding fish oil (FO) into an oil blend still remains to be explored. Fish oil contains a high amount of polyunsaturated fatty acids which would alter the physicochemical properties of the blend upon its addition. Furthermore, rearrangement of the fatty acids positions in the TAG backbone, either by chemical or enzymatic interesterification, would cause further changes to the oil blend properties.

An experiment was formulated using Modde 6.0 software developed by Umetrics in order to have a better understanding on the effect of blending FO with palm stearin (PS) and palm kernel oil (PKO). Ten oil blends were prepared and interesterified by Novozyme TL IM lipase (*Thermomyces lanuginosa*), a sn-1, 3 specific lipase. The range of oil in the blend are as follows: PS, 60-90%; PKO, 10-40%; FO, 0-10%. The objective of using the specific lipase is to keep the omega-3 fatty acids at the sn-2 position and to have short or medium chain fatty acids at the outer positions, as illustrated in figure 1. The actual scheme is actually more complex since the blend consists of a wide range of fatty acids and the TAG molecules could comprise of a mixture of fatty acids. Therefore, figure 1 only shows a simplified version of the actual reaction scheme. The reaction was conducted using a continuous packed bed reactor with a residence time of 2 hours. Samples before and after enzymatic interesterification were analyzed for TAG content by GC, SFC, dropping point and FFA.



Fig 1. Reaction scheme of sn-1,3 specific enzymatic interesterification. S, M and L represent short, medium and long chain fatty acids respectively; E, EPA; D, DHA.

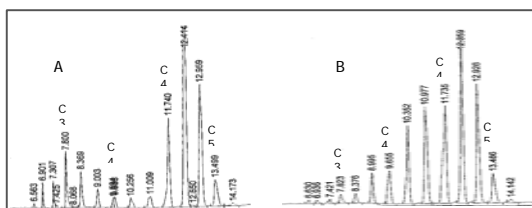


Fig 2. TAG chromatogram of PS/PKO/FO (72.5/22.5/5, wt%) blend before (A) and after (B) interesterification

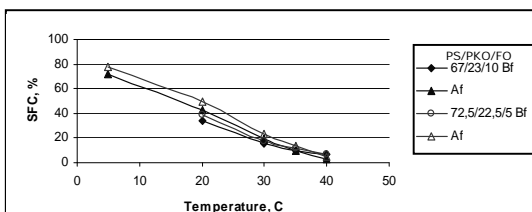


Fig 3. SFC profile. Enzymatic ie increased the SFC (at 5 to 30 °C), as shown in before (Bf) and after (Af) ie.

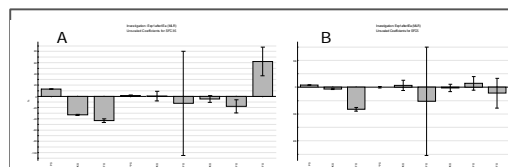


Fig 4. Coefficient plot for SFC 35 °C (A) and SFC 5 °C (B) FO shows the strongest effect on SFC 5 °C whilst the effect of the other oils become diminish.

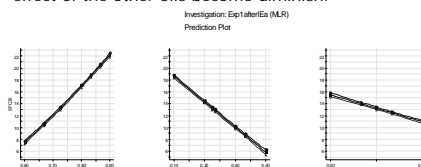


Fig 5. Effect of PS, PKO and FO on SFC at 35 °C

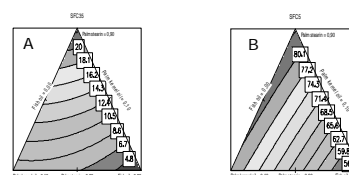


Fig 6. Contour plot of SFC at 35 °C (A) and 5 °C (B).

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Summary

Naturally, omega-3 FAs are located at the sn-2 position and by employing a sn-1, 3 specific lipase, the EFA should remain at the original position. After ie, the amount of TAGs containing only either short or long chain FA were decreased whilst those containing a mixture of FA and of intermediate length were increased (Fig 2). The reaction also led to a higher SFC in the interesterified products compared to the stock oil blend (Fig 3). However, the former had a sharper melting point than the latter. Even though FO was only a minor component (10%), yet it has a strong influence on the SFC (Figs 4 and 5). From Fig 6, PS/PKO/FO (55/15/30, wt%) is predicted to have a similar SFC profile with a commercial table margarine.



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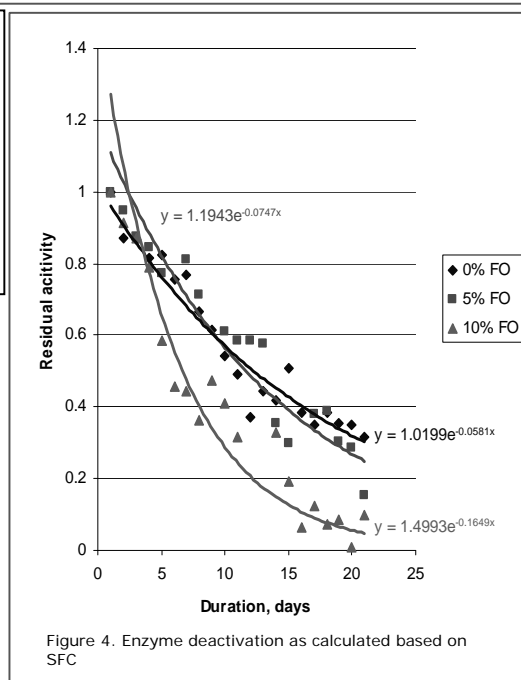
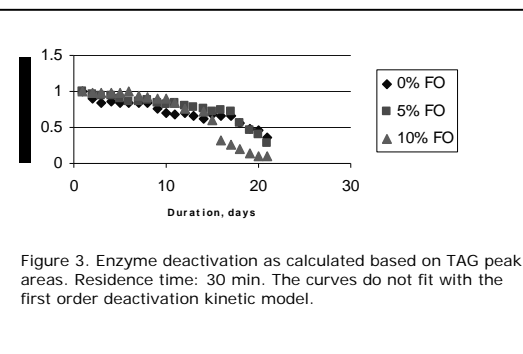
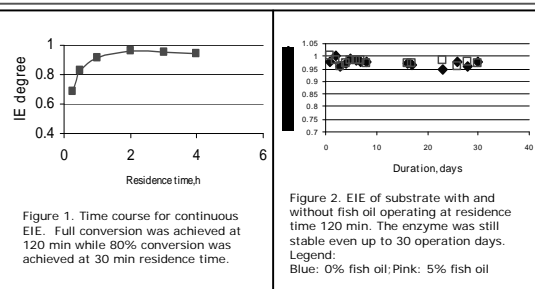
Effect of fish oil on enzyme stability during enzymatic interesterification

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Background

Nutritional value of fats and oils can be further enhanced by the advent of enzymatic interesterification (EIE) technology. Incorporation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into triacylglycerol (TAG) of lipids is one of the examples of the application of such technology. EPA and DHA are categorized as omega-3 fatty acids, and also known as essential fatty acids (EFA) due to their beneficial effect to human health. The addition of fish oil (FO) would also lead to a change in the physicochemical property of a lipid. A lot of studies have been conducted with regard to this area. Fish oil, a rich source of EPA and DHA, contains a very high amount of polyunsaturated fatty acids (PUFA). Oxidation products of PUFA have been well established to be detrimental to enzyme stability. This paper would address the effect of residence time and fish oil concentration on enzyme stability. Enzyme deactivation was monitored by the change in TAG and solid fat content at 20 °C.

A blend of palm stearin (PS) and palm kernel oil (PKO), 75/25% w/w, containing 0, 5 and 10% FO (w/w) was used as a substrate. A continuous interesterification reaction was conducted by a packed bed reactor using a 200 mm X 15 mm i.d. stainless steel column filled with Lipzyme TL IM lipase (*Thermomyces lanuginosa*). Substrate flow rate was adjusted to give residence time of 120 min and 30 min to see the effect of full and 80% conversion respectively, as shown in Figure 1, on enzyme stability. The reaction was conducted continuously for 30 days and 21 days running at 120 min and 30 min residence time respectively. Samples before and after enzymatic interesterification were analyzed for SFC and TAG content by HPLC.



Summary

1. Even though the enzyme was stable when operating at 120 min residence time, this would lead to a low productivity.
2. The blend containing 10% FO showed the most rapid decrease in enzyme activity when the system was operating at 30 min residence time. A sharp decrease was observed after 16 days.
3. From Figure 4, the deactivation rate of the blend containing 10% FO is 3 times higher than the one without FO. Enzyme's half-life: 0% FO, 12 days; 5% FO, 9 days; 10% FO, 4 days.
4. A 'two-step series deactivation model' would be more appropriate in monitoring the enzyme deactivation by TAG since the rate was not constant.
5. TAG would be a better tool than SFC in monitoring the deactivation since EIE does not only



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Structured Triacylglycerol of Palm-based Margarine by Enzymatic Interesterification

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Margarine can be produced either by blending, hydrogenation or interesterification (enzymatic or chemical). Previously, partial hydrogenation was a common method for margarine production. The process has been ceased from operation in margarine plant due to formation of trans fatty acid which is known to be carcinogenic. Chemical interesterification is also a popular method but disposal of nickel catalyst poses hazard to the environment. Enzymatic interesterification has gained a lot of attention lately due to the regiospecificity of lipases which enable the production of structured lipids (SL). SL can be designed to improve physical characteristics of fats, such as melting behavior or plasticity. Furthermore, enzymes are environmental friendly since they are biodegradable.

An experiment was formulated using Modde 6.0 software developed by Umetrics in order to have a better understanding on the effect of blending FO with palm stearin (PS) and palm kernel oil (PKO). Ten oil blends were prepared and interesterified by Novozyme TL IM lipase (*Thermomyces lanuginosa*), a sn-1, 3 specific lipase. The range of oil in the blend are as follows: PS, 60-90%; PKO, 10-40%; FO, 0-10%. The purpose of applying the specific lipase is to keep the omega-3 fatty acids at the sn-2 position and to have short or medium chain fatty acids at the outer positions, as illustrated in Figure 1. The former would become a source of essential fatty acid (EFA) after being absorbed through the intestinal wall whilst the latter would be consumed as a quick source of energy. The actual scheme is actually more complex since the blend consists of a wide range of fatty acids and the TAG molecules could comprise of a mixture of fatty acids. Therefore, Figure 1 only shows a simplified version of the actual reaction scheme. The reaction was conducted using a continuous packed bed reactor with a residence time of 30 minutes. Samples before and after enzymatic interesterification were analyzed for SFC and TAG content by GC. Enzyme deactivation rate was calculated based on SFC



Fig 1. Reaction scheme of sn-1,3 specific enzymatic interesterification. S, M and L represent short, medium and long chain fatty acids respectively; E, EPA; D, DHA.

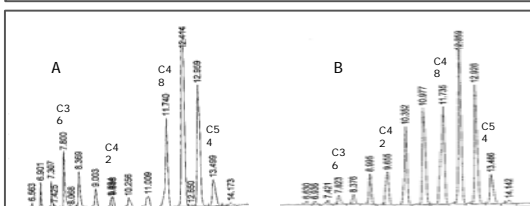


Fig 2. TAG chromatogram of PS/PKO/FO (72.5/22.5/5, wt%) blend before (A) and after (B) interesterification

Table 1. FAC of oils analyzed by GC

	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C20:5	C22:6
PKO	7.2	6.3	51.6	16.4	6.9	1.5	8.2	1.3					
POS	0.2	1.2	58.8	4.7	29.2	5.6	0.3						
FO			5.7	12.3	2.4	17.6	2.4	12.7	4.0	8.8	11.6	14.7	
Blend													
10/80/10	5.6	2.8	42.3	4.2	29.8	6.1	0.3	0.7	1.4	1.3	0.8		
23/67/10	11.5	4.9	38.2	3.6	26.4	5.7	0.2	0.4	1.6	0.8	1.2		

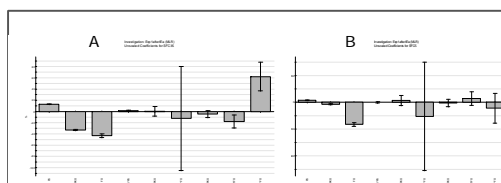


Fig 3. Coefficient plot for SFC 35°C (A) and SFC 5°C (B) FO shows the strongest effect on SFC 5 °C whilst the effect of the other oils is diminishing.

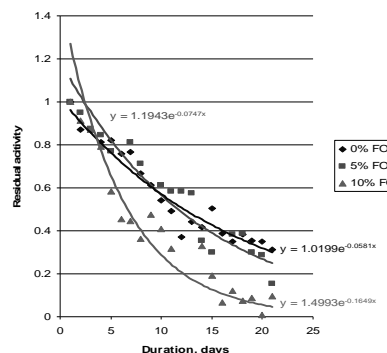


Figure 4. Enzyme deactivation as calculated based on SFC at 20 °C. Residence time: 30 min.



Acknowledgement
This study was funded by MPOB

Summary

Sn-1,3 specific lipase will preserve the EFA at the sn-2 position and esterify those at the outer positions. EIE product contains higher amount of intermediate TAG (C42-C46) as compared to the blend (Fig 2). The TAG contains a wide range of fatty acids as shown in Table 1. The reaction also led to a higher SFC in the interesterified products than the stock oil blend. However, the former had a sharper melting point than the latter. Even though FO was only a minor component (10%), yet it has a strong influence on the SFC (Fig 3). Deactivation rate of enzyme fed with oil blends containing 10% FO is three times faster than the blend without FO due to the high PUFA content in the former (Fig 4). Enzyme is very sensitive to substrate quality. High PUFA oil is easily oxidized which led to the fast deactivation rate of the enzyme.

